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Biochimica et Biophysica Acta 1711 (2005) 41–48
BIOCHIMICA ET BIOPHYSICA ACTA
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Electroneutral K^+/H^+ exchange in mitochondrial membrane vesicles involves Yol027/Letm1 proteins

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Received 25 November 2004; received in revised form 8 February 2005; accepted 28 February 2005
Available online 22 March 2005

Abstract

YOL027c in yeast and *LETM1* in humans encode integral proteins of the inner mitochondrial membrane. They have been implicated in mitochondrial K^+ homeostasis and volume control. To further characterize their role, we made use of submitochondrial particles (SMPs) with entrapped K^+- and H^+-sensitive fluorescent dyes PBFI and BCECF, respectively, to study the kinetics of K^+ and H^+ transport across the yeast inner mitochondrial membrane. Wild-type SMPs exhibited rapid, reciprocal translocations of K^+ and H^+ driven by concentration gradients of either of them. K^+ and H^+ translocations have stoichiometries similar to those mediated by the exogenous K^+/H^+ exchanger nigericin, and they are shown to be essentially electroneutral and obligatorily coupled. Moreover, $[K^+]$ gradients move H^+ against its concentration gradient, and vice-versa. These features, as well as the sensitivity of K^+ and H^+ fluxes to quinine and Mg^{2+}, qualify these activities as K^+/H^+ exchange reactions. Both activities are abolished when the yeast Yol027p protein is absent (*yol027*Δ mutant SMPs), indicating that it has an essential role in this reaction. The replacement of the yeast Yol027p by the human Letm1 protein restores K^+/H^+ exchange activity confirming functional homology of the yeast and human proteins. Considering their newly identified function, we propose to refer to the yeast *YOL027c* gene and the human *LETM1* gene as *yMKH1* and *hMKH1*, respectively.

Keywords: Submitochondrial particle; BCECF; PBFI; Cation/proton exchange; Inner mitochondrial membrane; K^+/H^+ antiport

1. Introduction

Mitochondria are surrounded by two membranes, an outer membrane and an inner membrane, which differ in their permeability to small molecules. The trafficking of metabolites and ions through the outer membrane is believed to be mediated primarily by the mitochondrial porin, forming the non-selective voltage-dependent anion channel VDAC [1]. The passage of small molecules through the inner membrane, in contrast, has been shown to be mediated by a variety of substrate-specific transport systems [2,3].

In addition to this protein-mediated transit, ions can pass membranes by diffusive processes. This passive permeability of membranes is generally low, but the high, inside negative membrane potential $\Delta\psi$ of mitochondria constitutes a powerful driving force for diffusive influx of cations, notably of the highly abundant K^+, and renders passive permeability a process of high physiological significance. It tends to increase matrix osmolarity, causing influx of water and swelling of the organelle. If unchecked, cation leaks can result in the disruption of mitochondrial morphology and eventually lysis of the organelle [2–4].

Cation/proton antiporters have first been postulated by P. Mitchell [5] for compensating this cation leakage and controlling mitochondrial volume. Mammalian cells appear to have two independent monovalent cation/proton antiport

Abbreviations: DCCD, Dicyclohexylcarbodiimide; SMP, submitochondrial particle; $\Delta\Psi$, membrane potential

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doi:10.1016/j.bbamem.2005.02.018

systems, an overt Na^+-selective and a latent non-selective antiporter. Both antiporters are likely to catalyze electroneutral exchange of a cation for H^+. The non-selective antiporter is distinct from the Na^+-selective one in that it transports Na^+, K^+, Li^+, Rb^+ as well as Cs^+ with K_m values varying between 30 and 120 mM. It is inhibited by quinine, propanolol or DCCD and it is allosterically regulated by Mg^{2+} [4,6]. Given that cytosolic K^+ concentrations (150 mM) are much higher than Na^+ concentrations, the non-selective antiporter can be expected in vivo to mediate K^+/H^+ rather than Na^+/H^+ exchange. Hence, it is commonly referred to as K^+/H^+ antiporter or exchanger.

The characterization of K^+/H^+ exchange activities have mostly been based on the determination of potassium acetate (KOAc)-induced, passive swelling of non-respiring, isolated mitochondria which is accompanied by a change in light scattering of the organelles [2]. Cation fluxes have also been observed in submitochondrial particles (SMPs) with entrapped H^+-sensitive fluorescent dyes [7]. Finally, K^+/H^+ activity was reconstituted in liposomes by the use of a DCCD-binding protein fraction from mammalian mitochondria. Electroneutral K^+/H^+ exchange activity was attributed to a 82 kDa protein band [8]. This work has not resulted in a molecular characterization of the K^+/H^+ exchanger yet.

We have characterized a nuclear gene, *YOL027c*, of the yeast *Saccharomyces cerevisiae* as encoding an integral protein of the inner mitochondrial membrane [9]. Due to its involvement in mitochondrial morphology, *YOL027c* has previously been named *MDM38* [10]. Mutants lacking this gene (*yol027*Δ) fail to grow on non-fermentable substrate, indicating that they are mitochondrially defective. They are disturbed in mitochondrial K^+ homeostasis and volume control. Isolated *yol027*Δ mitochondria are refractory to KOAc-induced swelling which is indicative of a defect in K^+/H^+ exchange activity. The expression of the human homologue of Yol027p, named Letm1, restores KOAc-induced swelling as well as growth on non-fermentable substrates [9]. The *LETM1* gene is part of a large hemizygous deletion in humans, causing the Wolf-Hirschhorn disease [11,12].

Here we prepared submitochondrial particles (SMPs) from a wild-type yeast strain and observed K^+ and H^+ translocations into and out of these SMPs by use of the entrapped K^+- and H^+-sensitive fluorescent dyes PBFI and BCECF, respectively. Potassium and proton movements, driven by their concentration gradients across the SMP membrane, were observed as expected if resulting from $[K^+]$- and $[H^+]$-driven electroneutral K^+/H^+ exchange. Both were sensitive to known inhibitors of K^+/H^+ exchange. $[K^+]$- and $[H^+]$-driven translocation of potassium and protons were essentially abolished in SMPs prepared from a strain deleted for *YOL027*c (*yol027*Δ mutant). The expression of the human Letm1 protein in *yol027*Δ mutant yeast restored K^+/H^+ exchange activity of SMPs.

2. Material and methods

2.1. Yeast strains, plasmids and media

DBY747 wild-type (ATCC no. 204659) and DBY747 *yol027*Δ [9] were grown in YPD (1% yeast extract, 1% peptone, 2% dextrose) overnight to stationary phase. Synthetic selective media were used to grow DBY747 *yol027*Δ transformants carrying the plasmid pVT103-U [13] either without insert, with the human *LETM1* cDNA [9] or with the yeast *YOL027c* open reading frame. Both inserts were under the ADH promoter.

2.2. Preparation of PBFI- or BCECF-loaded submitochondrial particles (SMPs)

Yeast spheroblasts were lysed in 0.6 M sorbitol, 10 mM Tris/HCl, pH 7.4, buffer and isolated by differential centrifugation as described before [14]. In order to obtain mitoplasts, the mitochondrial pellet was resuspended in low-osmolarity buffer (10 mM Tris–HCl, pH 7.4, 0.1 M sorbitol). After 20-min incubation on ice, the organelles were collected by centrifugation (40,000 × *g*, 10 min, 4 °C) and resuspended in sucrose buffer (250 mM sucrose/10 mM Tris–HCl, pH 7.4). As shown by Nowikovsky et al. [9], resulting mitoplasts essentially lacked the outer mitochondrial membrane. To obtain SMPs, this mitoplast suspension was pulse-sonified three times for 60 s with 80% intensity in a Bandelin sonicator UW70/GM70 for a total of 3 min while keeping the samples in ice water. After removing unbroken mitochondria (centrifugation at 10,000 × *g*, 10 min, 4 °C), SMPs were collected by centrifugation of the supernatant at 100,000 × *g* for 1 h, 4 °C.

2.3. Loading of SMPs with dyes and fluorescence measurements

The SMP pellet was resuspended in 1 ml of sucrose buffer (1 mg protein per ml) containing either the K^+-sensitive or H^+-sensitive fluorescent dye (75 μM PBFI salt or 50μM BCECF salt, respectively). To entrap these dyes in the SMPs, the suspensions were again sonified as described above. SMPs were finally collected by centrifugation, washed once and suspended in sucrose buffer to a final concentration of 0.05 mg protein per ml.

The excitation wavelengths for PBFI were set at 340 and 380 nm and for BCECF at 440 nm and 490 nm while the emission wavelengths were set at 500 nm (PBFI) and 535 nm (BCECF). Fluorescence was recorded with a Perkin Elmer LS-55 spectrofluorometer with Fast Filter Accessory (FFA). All measurements were done in 3 ml cuvettes containing 2 ml of SMP suspension with stirring at 25 °C. As revealed from measuring fluorescence in post-SMP supernatants, there was no apparent leakage of the fluorescent dyes from the loaded SMPs during a period of 2 h.

Nigericin (10 μM) was added 5′ before the measurement of fluorescence. For inhibition experiments, SMPs were incubated with 3 mM Mg^{2+}, 10 μM quinine, and 10 mM DCCD 10 min prior to the measurements.

To calibrate $[K^+]$, KCl was added to sucrose buffer with 7.5 μM PBFI to final K^+ concentrations ranging from 0 mM to 200 mM. Fluorescence signals (x) were plotted against $[K^+]$ of the buffers. This resulted in a calibration curve which can be described with the formula: $[K^+]=y=0.0914\ln(x)+1.8024$. The calibration of BCECF signals was performed by adding BCECF (5 μM) to sucrose buffers with pHs ranging from 6.5 to 8.5. The fluorescence signals of BECEF were plotted against the pHs of the buffers. This resulted in the formula $pH=y=-3.5129x+9.2963$. The formulas were used to estimate the K^+ concentrations and the pH inside SMPs.

Each experiment was repeated at least three times with different SMP preparations. The obtained fluorescence changes were used to calculate $[K^+]$ and pH values with the help of the formulas stated above. $[K^+]$ and pH values given in the text are means plus standard deviations of at least three different concentration calculations.

2.4. Changes in $\Delta\psi$ of SMPs as determined by $DiBAC_4(3)$

For the assessment of membrane potential in SMPs, we used $DiBAC_4(3)$, an anionic oxonol dye [15] that enters depolarized membranes. Potential-dependent decreases in $DiBAC_4(3)$ fluorescence are in the order of 1% per mV (manufacturer's protocol).

$DiBAC_4(3)$ was added to SMPs (2 mg protein per ml) to a final dye concentration of 100 nM. SMPs were incubated for 30 min at 25 °C, suspended to a protein concentration of 0.05 mg/ml and immediately used for measurement.

The excitation wavelength for $DiBAC_4(3)$ was set at 488 nm while the emission was recorded at 510 nm. Data collection and analysis were performed with Perkin Elmer LS-55.

2.5. Chemicals

PBFI (potassium binding benzofuran isophtalate) tetraammonium salt and BECEF (2′, 7′-bis-(2-carboxyethyl)-5 and 6-carboxyfluorescin) tetraammonium salt were purchased either from Molecular Probes (The Netherlands) or Mobitech (Germany). $DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid) trimethine oxonol) was purchased from Molecular Probes (The Netherlands).

All chemicals were purchased from Sigma. Nigericin and DCCD were diluted in 70% ethanol, valinomycin in DMSO and quinine in ultra-pure water. Solvent controls showed neither a change in the fluorescence signals nor an influence on the stability of the SMPs. Quinine showed an enhancement of the fluorescence signal which was subtracted from the data.

3. Results

3.1. Fluorescence determination of $[H^+]$ and $[K^+]$ in SMPs

As previously shown, our preparation of SMPs from yeast mitoplasts resulted in vesicles which were virtually devoid of outer membranes, and their vast majority exposed the matrix side of the inner membrane, i.e. they had an inside-out membrane orientation [9]. Preparing SMPs enabled us to generate identical, nominally cation-free milieus inside and outside of the vesicles and to entrap $K^+(Na^+)$- or H^+-sensitive dyes PBFI and BCECF. The fluorescence intensities of these dyes were 46 times (PBFI) and 15 times (BCECF) higher than the autofluorescence of the unloaded SMPs (data not shown). During several hours of incubation, the dyes stayed entrapped in the SMPs, and there was no indication for rupture of SMPs if ion concentrations or pH were changed on the outside of the SMPs.

With increasing or lowering the pH outside of the vesicles (pH_o), we generated outward- or inward-directed proton gradients ($\Delta[H^+]$) and by raising outside K^+ concentrations ($[K^+]_o$), we obtained inward-directed K^+ gradients ($\Delta[K^+]$). The resulting changes in H^+ and K^+ concentrations inside SMPs ($[K^+]_i$, $[H^+]_i$) were monitored as changes in PBFI and BCECF fluorescence and calculated into concentrations with the help of the formulas obtained from calibration curves. SMPs were incubated in sucrose buffers lacking any respiratory substrate and ATP. Additionally, antimycin A and oligomycin, blocking respiration and ATPase activity, respectively, were present in the buffers.

3.2. $\Delta[K^+]$ as well as $\Delta[H^+]$ elicit passive changes in $[K^+]_i$ and $[H^+]_i$

SMPs were prepared from wild-type yeast mitochondria in nominally K^+-free buffer with equal pH_o and pH_i of 7.4. The addition of KCl to yield a $[K^+]_o$ of 150 mM resulted in a rapid change of $[K^+]_i$ to a steady-state level of 101.2 mM (Fig. 1A). This was paralleled by an alkalinization of the interior of SMPs (from pH 7.4 to 7.84) reflecting massive extrusion of protons (Fig. 1B). Alkalinization was also observed when KCl, NaCl or LiCl, each at 150 mM, was used to drive the reaction (Fig. 1C).

$\Delta[K^+]$-driven K^+ uptake of SMPs came to a halt before the full equilibration of $[K^+]_o$ and $[K^+]_i$ ($[K^+]_o$ 150 mM versus $[K^+]_i$ 101.2 mM) and it generated an inward-directed $\Delta[H^+]$ (pH_o 7.4 versus pH_i 7.84; cf. legend to Fig. 1). This was to be expected if the intrinsic activity of SMPs mediated K^+/H^+ exchange, generating a $\Delta[H^+]$ which opposed further exchange activity and thus full equilibration of $[K^+]_o$ and $[K^+]_i$.

Next, we asked if a $[H^+]$ gradient would equally drive the translocation of both H^+ and K^+. Therefore, we prepared SMPs with $[K^+]_o$ and $[K^+]_i$ equally set at 10 mM and then

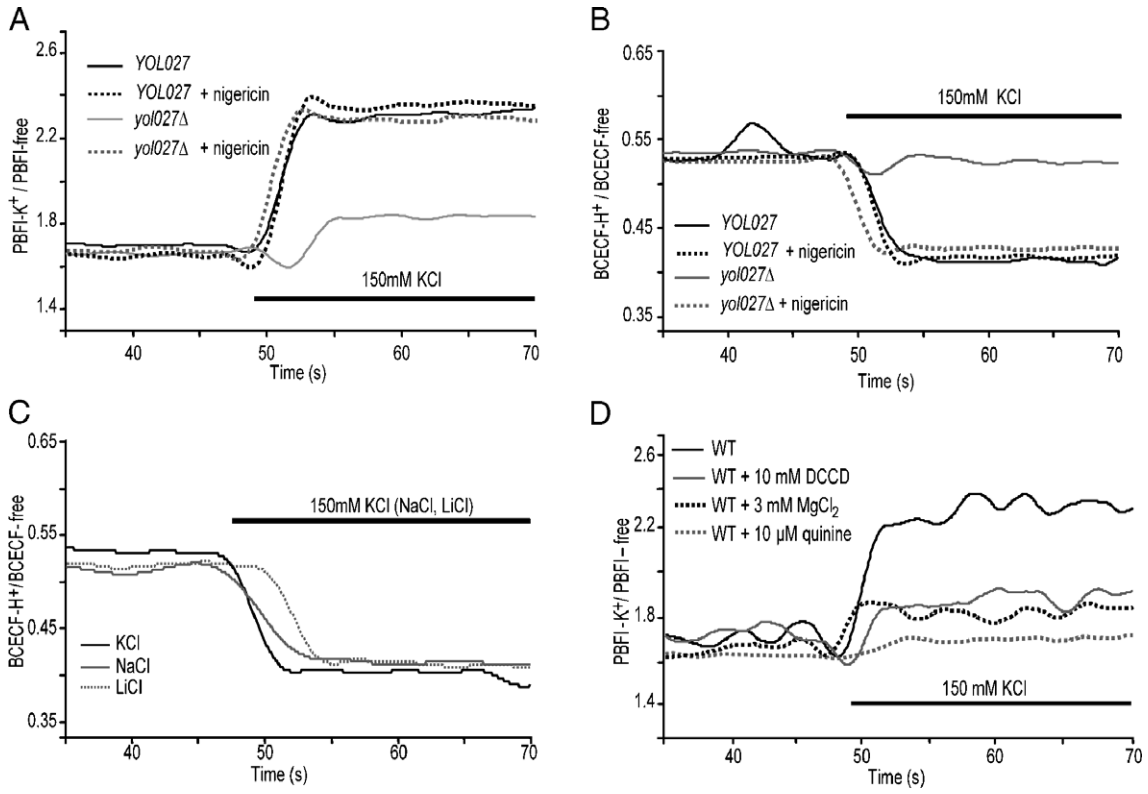

Fig. 1. $[K^+]$ ($[Na^+]$ $[Li^+]$)-driven changes of $[K^+]_i$ and $[H^+]_i$ in SMPs. Submitochondrial particles (SMPs) prepared from wild-type (*YOL027*) and mutant (*yol027*Δ) cells with entrapped K^+-sensitive PBFI or H^+-sensitive BCECF, with equal pH_o and pH_i (7.40) and with nominally alkali-free buffer inside and outside. Ratios of K^+-bound or H^+-bound to -unbound dyes were recorded at 25 °C at resting conditions and upon the addition of KCl to final $[K^+]_o$ as indicated and in the absence or presence of stimulating or inhibiting factors as indicated. Concentrations given below were based on calibration formulas for PBFI and BCECF fluorescence signals (cf. Materials and methods). (A) Relative increase of $[K^+]_i$ observed in wild-type SMPs (*YOL027*) and in mutant SMPs (*yol027*Δ) with or without the addition of nigericin. At 150 mM, $[K_o]$ steady-state $[K_i]$ was calculated to be 101.2 mM (±5.8) and 104.0 (±3.0) in wild-type SMPs without and with nigericin, respectively, and 5.5 mM (±2.3) and 98.4 mM (±1.9) in *yol027*Δ SMPs without and with nigericin, respectively. (B) Relative increase of pH observed with wild-type SMPs (*YOL027*) and mutant SMPs (*yol027*Δ) with or without the addition of nigericin. Steady-state pH_i were calculated to be 7.84 and 7.85 in wild-type SMPs without and with nigericin, respectively, and 7.45 and 7.82 in *yol027*Δ SMPs without and with nigericin, respectively. (C) Relative decrease in $[H^+]_i$ observed with wild-type (*YOL027*) driven either by 150 mM KCl, 150 mM NaCl or 150 mM LiCl. Steady-state pH_i were calculated to be 7.84 (KCl), 7.79 (NaCl) and 7.80 (LiCl). (D) Inhibitory effect of DCCD, $MgCl_2$ or quinine on the Δ$[K^+]$-driven increase of $[K^+]_i$ in wild-type SMPs. Steady-state $[K_i]$ were calculated to be 1.7, 1.2 and 1.3 in the presence of 3 mM $MgCl_2$, of 10 µM quinine and of 10 mM DCCD, respectively.

either acidified or alkalinized the buffer. A decrease of pH_o from 7.5 to 6.5, generating an inward-directed Δ$[H^+]$, elicited an efflux of K^+ (Fig. 2A), while an increase in pH_o (from 7.5 to 8.5) resulted in K^+ influx (Fig. 2B). H^+ translocations occurred with similar kinetics as K^+ translocations, but in opposite directions (data not shown). Interestingly, an outward directed Δ$[H^+]$ served as a driving force for the accumulation of K^+ inside the SMPs against its concentration gradient (from 10 mM to 16.4 mM), which is fully compatible with a K^+/H^+ exchange reaction. The addition of nigericin, a K^+/H^+ exchanger, did not result in further significant translocations of K^+ and H^+ (Fig. 1A, B) indicating that the intrinsic system of the SMPs and the exogenous K^+/H^+ exchanger led to similar $[H^+]_i$ and $[K^+]_i$ values.

3.3. Reduction of K^+ and H^+ translocation by inhibitors

The known inhibitors of mitochondrial K^+/H^+ exchange, quinine and DCCD, were found to strongly inhibit Δ$[H^+]$- as well as Δ$[K^+]$-driven K^+ and H^+ translocation by more than 95% (Figs. 1D and 2A, B). Despite of a high Δ$[K^+]$ or Δ$[H^+]$ PBFI signals stayed constant over an extended period of time. This revealed that neither fluorescent dyes nor K^+ or H^+ leaked into or out of the SMPs at any significant rate. Mg^{2+} has repeatedly been reported to inhibit K^+/H^+ exchange in mammalian mitochondria, but not in the mitochondria of two yeast strains [17,18]. However, with mitochondria of the strain used here (DBY747) we found pronounced Mg^{2+}-sensitivity of KOAc-induced swelling, which reflects K^+/H^+ exchange activity [9], and we observed here that Mg^{2+} added to SMPs exerted a strong inhibition of K^+ and H^+ fluxes driven either by Δ$[H^+]$ or Δ$[K^+]$ (Figs. 1 and 2).

3.4. Effect of YOL027 deletion

Most importantly, SMPs prepared from mutant *yol027*Δ mitochondria failed to exhibit the pronounced changes in Δ$[H^+]$ or Δ$[K^+]$ as observed in wild-type SMPs (Figs. 1A, B and 2A, B). Actually, there was no significant, immediate

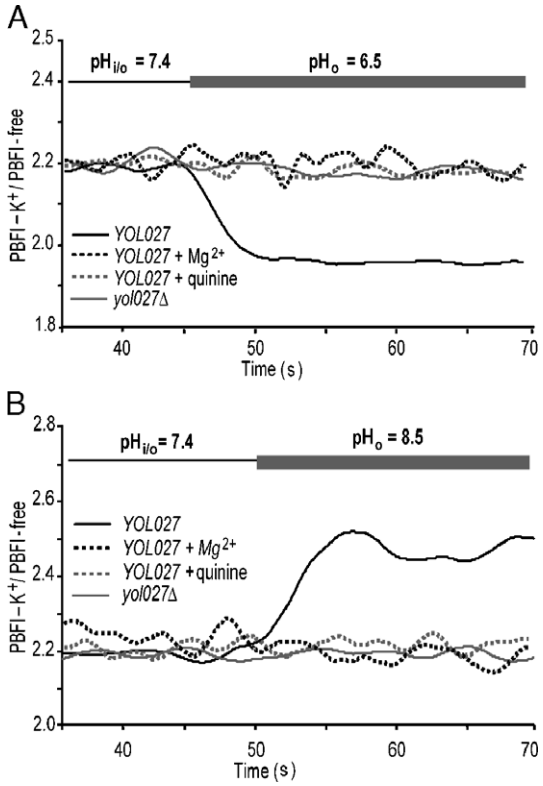

Fig. 2. Δ[H$^+$]-driven changes of [K$^+$]$_i$ and in SMPs. SMPs were prepared to have the K$^+$-sensitive dye PBFI entrapped and equal pH$_o$ and pH$_i$ (7.5) as well as equal [K$^+$]$_o$ and [K$^+$]$_i$ (10 mM). The relative increase of [K$^+$]$_i$ was recorded upon a decrease in pH$_o$ from 7.4 to 6.5 (A) or upon an increase in pH$_o$ from 7.4 to 8.5 (B), in wild-type SMPs (*YOL027*), with or without the addition of Mg^{2+} or quinine as indicated, and in mutant SMPs (*yol027*Δ). Steady-state [K$_i$] of wild-type SMPs was calculated to have decreased from 10 mM to 1.3 mM (A) and increased from 10 mM to 16.4 mM (B).

change in [H$^+$]$_i$ or [K$^+$]$_i$, except for a modest increase in [K$^+$]$_i$ when [K$^+$]$_o$ was raised to 150 mM. This increase in [K$^+$]$_i$ was about 5% of that seen with wild-type SMPs. It was significantly inhibited by 10 mM DCCD or 3 mM Mg^{2+}, but not by quinine (data not shown). It remains to be determined if this reflects K$^+$ transport activity independent of the Yol027 protein or simply passive permeability of the SMP membranes.

Δ[Na$^+$] or Δ[Li$^+$] equally failed to exhibit H$^+$ extrusion in *yol027*Δ SMPs (not shown). K$^+$, Na$^+$ and Li$^+$ translocations across the SMP membrane thus were equally abolished when the Yol027 protein was absent.

Like with wild-type SMPs, we did not observe any pronounced leakage of the mutant SMPs, indicating that the absence of the Yol027 protein from these SMPs had no apparent effect on the stability of the SMPs or their permeability, except for cation and proton movements. This was further supported by our finding that the addition of the K$^+$/H$^+$ ionophore nigericin to mutant SMPs restored K$^+$ and H$^+$ translocation to levels as mediated by the intrinsic activity of wild-type SMPs (Fig. 1A and B).

The expression of the human *YOL027* homolog, *LETM1*, in *yol027*Δ mutant yeast has previously been shown to

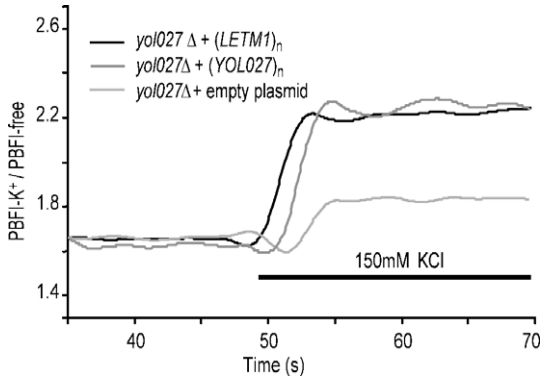

Fig. 3. Human Letm1p is a functional homolog of yeast *Yol027p*. SMPs were prepared from mitochondria of the yeast mutant *yol027*Δ carrying either the multi-copy plasmid pVT103-U empty or containing the yeast *YOL027* open reading frame (*YOL027*)$_n$ or the human *LETM1* gene (*LETM1*)$_n$. SMPs were treated as in Fig. 1 and PBFI fluorescence ratios were determined.

partly compensate for the growth defect of yeast cells [9]. As shown in Fig. 3, K$^+$ translocation was restored in SMPs prepared from *LETM1*-complemented *yol027*Δ mutant yeast cells as well as in SMPs of mutant cells complemented by the yeast *YOL027* expressed from the same multi-copy

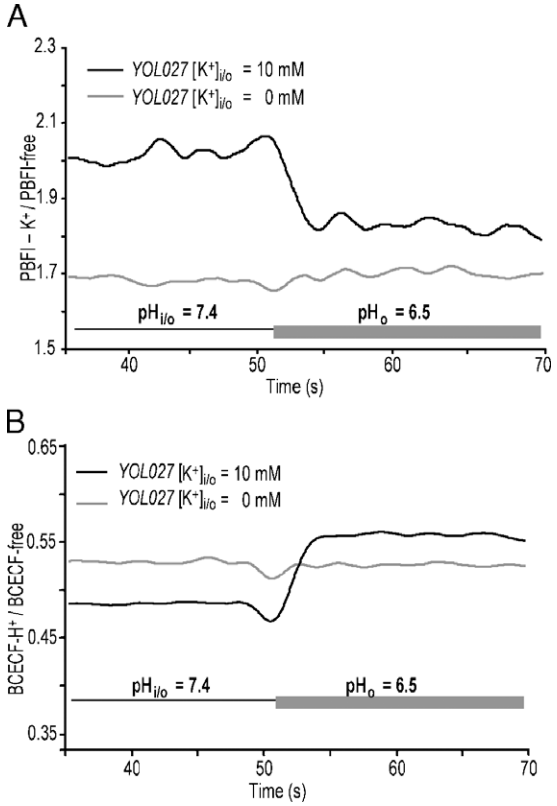

Fig. 4. Dependence of Δ[H$^+$]$_o$-driven H$^+$ fluxes on the presence of K$^+$. PBFI- or BCECF- loaded wild-type SMPs were prepared in sucrose buffer to have equal pH$_o$ and pH$_i$ (7.4), and equal [K$^+$]$_o$ and[K$^+$]$_i$, either 10 mM or nominally 0 mM. Upon changing pH$_o$ from 7.4 to 6.5, relative changes in [K$^+$]$_i$, (A) or pH$_i$ (B) were recorded. Changes in fluorescence signals were indicative of a decrease of [K$_i$] from 10 mM to 1.3 mM and of pH$_i$ from 7.4 to 7.28.

plasmid. The *LETM1*-mediated K^+ translocation was sensitive to quinine and Mg^{2+} (not shown). These findings underline the functional homology of the yeast Yol027p and the human Letm1 protein.

3.5. Obligatory exchange of H^+ and K^+

In order to see to which extend H^+ and K^+ translocations across SMP membranes were interdependent, we prepared SMPs in nominally K^+-free buffer with pH_i and pH_o 7.4 and generated an inward directed ΔpH by acidification of the exterior of the SMPs (pH_o 6.5). As shown in Fig. 4A and B, in the absence of K^+ inside and outside of SMPs, there was no significant change in pH. This showed that the movement of H^+ into or out of SMPs occurred only in the presence of K^+. The result clearly showed the interdependence of H^+ and K^+ translocations and strongly supported the notion that they were mediated by an exchanger.

We have also studied the dependence of K^+ translocation on pH. To this end we used a high $\Delta[K^+]$ first with no $\Delta[H^+]$ (equal pH_o and pH_i of 7.4), second with an inward directed $\Delta[H^+]$ (pH_o of 6.5 or 7.0) and third with an outward-directed

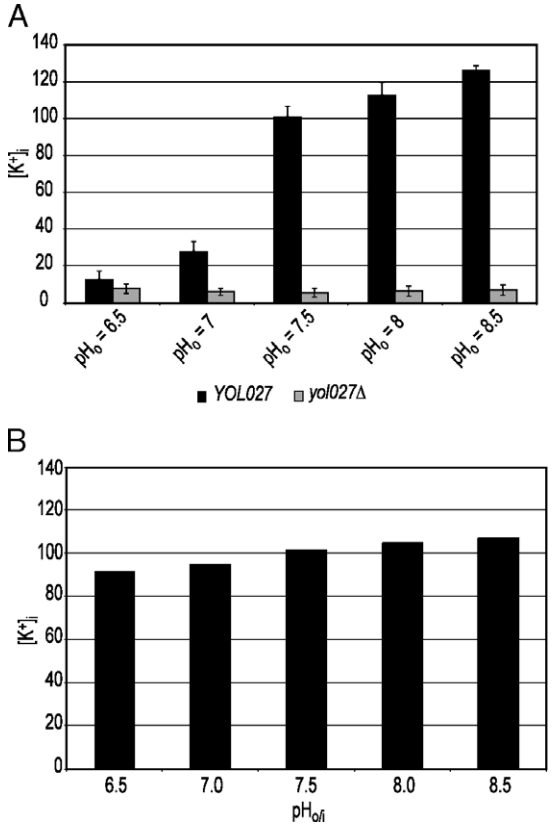

Fig. 5. Competitive inhibition of K^+/H^+ exchange by $[H^+]$. (A) Wild-type SMPs and mutant *yol027*Δ SMPs were prepared in nominally alkali-free sucrose buffer to have pH_i of 7.5 and pH_o ranging from 6.5 to 8.5. Steady-state $[K^+]_i$ values were determined upon raising $[K^+]_o$ to 150 mM. The bars represent means and standard deviations of at least 3 independent measurements. (B) Wild-type SMPs were prepared in sucrose buffers with identical pH_i and pH_o ranging from 6.5 to 8.5. Steady-state $[K^+]_i$ values were determined upon raising $[K^+]_o$ to 150 mM.

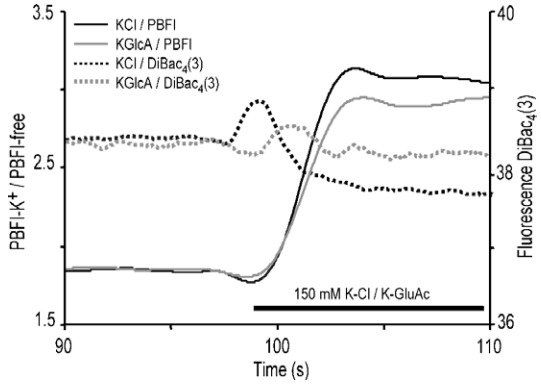

Fig. 6. Electroneutral K^+ influx into SMPs. Wild-type SMPs were prepared to have equal pH_i and pH_o (7.4) in nominally alkali-free sucrose buffer. One part of the SMPs was loaded with PBFI, the second one was incubated with $DiBAC_4(3)$. Either K-chloride or K-gluconate was added to final concentrations of 150 mM. The addition of K-chloride and K-gluconate resulted in a $[K^+]_i$ of 101.2 ± 5.8 mM and of 103.3 ± 3.0 mM, respectively. $DiBAC_4(3)$ fluorescence decreased within a few seconds after K-chloride addition by about 3%, equivalent to an increase in $\Delta\psi$ of 3 mV. There was no significant, short-term change in $DiBAC_4(3)$ fluorescence upon the addition of K-gluconate.

$\Delta[H^+]$ (pH_o of 8.0 or 8.5). As shown in Fig. 5A, an inward-directed $\Delta[H^+]$ strongly inhibited K^+ uptake by more than 90% while an outward directed $\Delta[H^+]$ slightly enhanced the K^+ influx.

While this result is fully consistent with a competitive inhibition of a K^+/H^+ exchange reaction, it does not exclude the possibility of an allosteric effect on the exchanger as it had been reported previously [6,16]. To determine if such an allosteric effect contributed to the pH dependence observed here, we studied the $\Delta[K^+]$-driven uptake of K^+ into SMPs with initial pH_o and pH_i set equal in a range from 6.5 to 8.5, i.e. we eliminated possible competitive effects of H^+ extrusion by $\Delta[H^+]$. As a result, we observed a mild decrease of K^+ uptake with decreasing pH (Fig. 5B). While this may have reflected allosteric effects on the protein(s) mediating K^+ and H^+ translocation, it contributed little to the inhibition resulting from lowering pH_o (Fig. 5A). Accordingly, we conclude that $\Delta[K^+]$-driven uptake of K^+ into SMPs is essentially opposed by inside-directed $\Delta[H^+]$ in a competitive manner.

3.6. Electroneutral K^+/H^+ exchange

K^+/H^+ exchange activities are consistent with the electroneutrality and independence of the potassium salt used. Measuring $\Delta[K^+]$-driven K^+ uptake with various potassium salts resulted in near identical changes in $[K^+]_i$ of wild-type SMPs (data no shown). Most remarkably, K^+ uptake occurred at a standard rate when the membrane impermeant anion gluconate was used instead of a permeant anion (Fig. 6). Thus, the K^+ uptake by SMPs is not linked to the uptake of an anion.

For the determination of changes in membrane potential we made use of $DiBAC_4(3)$ in parallel to measurements of

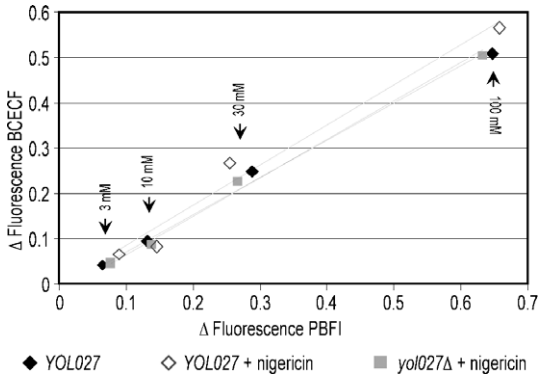

Fig. 7. Comparison of Δ[K^+]-driven changes in PBFI versus BCECF fluorescence mediated either by the endogenous system or by nigericin. Preparations of SMPs from wild-type and *yol027*Δ mutant cells were prepared as described in Fig. 1, either without nigericin (wild-type) or with nigericin (wild-type and mutant). In separate experiments, KCl was added to final concentrations of 3, 10, 30 or 100 mM and fluorescence ratios PBFI-K^+/PBFI and BCECF-H^+/BCECF were determined and plotted on the *x*-axis and *y*-axis, respectively.

Δ[K^+]-driven K^+ influx by use of PBFI (Fig. 6). We observed small, but noteworthy changes in membrane potential (4 to 6 mV), but the kinetics of these changes and of K^+ uptake or H^+ extrusion were quite different, indicating that they were not resulting from the same process. The changes in Δψ seen with permeant anions are likely the result of an electrogenic Cl^- uniport. This is supported by the fact that they were absent when the impermeant anion gluconate replaced the permeant chloride. Within the few seconds upon K^+ addition, while K^+ moved rapidly, changes in Δψ were below detection level (Fig. 6). Accordingly, K^+ influx into yeast SMPs occurred by an essentially electroneutral process.

Electroneutral exchange of K^+ and H^+ was also suggested by nearly identical steady-state levels of $[H^+]_i$ or $[K^+]_i$ resulting from Δ[K^+]-driven translocations mediated by the intrinsic system of wild-type cells or by nigericin in *yol027*Δ SMPs. This was not only observed when we applied 150 mM K^+ as a driving force (Fig. 1), but with $[K^+]_o$ values from 3 to 100 mM (Fig. 7). Apparently, both systems were equally efficient and had similar stoichiometries, which in case of nigericin is known to be a 1:1 exchange of K^+ and H^+ [19].

4. Discussion

K^+/H^+ antiport in mitochondria has been subject of many studies. Mainly, it has been characterized by passive swelling experiments and sensitivity to various drugs [2,3]. Experiments measuring both K^+ and H^+ translocations across the mitochondrial membrane, as reported here, are without precedent. They extend our knowledge on the mitochondrial K^+/H^+ exchange activity and identify yeast Yol027p and its human homolog Letm1 as essential components of the K^+/H^+ exchange system. We propose now to refer to these proteins as yMkh1 and hMkh1 (for mitochondrial K^+/H^+ exchange factor 1).

To study K^+ and H^+ translocations across the inner mitochondrial membrane, we introduced here the use of non-respiring, submitochondrial particles (SMPs) prepared from yeast mitochondria and entrapped fluorescent K^+ (and Na^+)- or H^+-sensitive dyes (PBFI or BCECF, respectively). Compared to intact mitochondria, SMPs facilitate transport studies. Their internal and external pH and cation concentrations can be designed at will and in a range allowing the use of fluorescent dyes, e.g. PBFI whose K_D of 11 mM does not allow any faithful determination of [K^+] in intact mitochondria. Matrix or membrane-associated factors, which may inhibit or modulate K^+/H^+ exchange in intact mitochondria, might dissociate during the preparation of SMPs. This can unmask activities of membrane proteins.

SMPs prepared from wild-type yeast mitochondria responded to an increase in $[K^+]_o$ by a rapid increase in $[K^+]_i$, which was accompanied by a concomitant decrease in $[H^+]_i$, consistent with an influx of K^+ and an efflux of H^+. Similarly, Δ[H^+] resulted in the rapid movement of protons down their concentration gradient, paralleled by K^+ movements into the opposite direction.

The data presented here is consistent with a K^+/H^+ exchange reaction, but not with independent K^+ and H^+ transport reactions. First, in spite of a high Δ[H^+], there was virtually no movement of protons, except when K^+ was added to the assay, indicating that both transports were obligatorily coupled. Second, the system transported H^+ as well as K^+ against their concentration gradients. It is difficult to visualize that a Δ[K^+] elicits K^+ influx via a uniporter and at the same time elicits proton movement against its gradient via another uniporter. Third, the massive influx of K^+ concomitant with H^+ efflux was not accompanied by any significant change in membrane potential. Furthermore, electroneutral K^+/H^+ exchange was also indicated by the fact that the exogenous K^+/H^+ exchanger nigericin and the endogenous system result in near identical electrochemical equilibration of [K^+] and [H^+]. Fourth, the reaction exhibited pronounced sensitivity to either quinine, DCCD or Mg^{2+} that are known inhibitors of the mitochondrial K^+/H^+ exchange. It transported Na^+ and Li^+ as well as K^+ in exchange for H^+, consistent with no or low selectivity towards monovalent cations [3,19,20]. All these features support the notion that the fluxes observed here were mediated by the electroneutral K^+/H^+ exchange system as it had been described previously based on more indirect measurements [3,19,20].

Yeast SMPs as used here had no significant activity of H^+ transport or H^+ leakage beside the described system for the obligatory exchange of K^+ against H^+. This is in contrast with a previous report on mammalian SMPs by Brierley et al. [21] who observed a spontaneous acid shift when non-respiring SMPs were suspended in acidic medium without addition of K^+. These authors provided circumstantial evidence for the presence of H^+ symport with chloride or another permeant anion. It remains to be seen if yeast mitochondria lack this symport or if it had been lost or inactivated during the preparation of SMPs. In any case, its

absence rendered our yeast SMP preparations particularly useful for the study of K^+/H^+ antiport.

In contrast to wild-type SMPs, mutant *yol027*Δ SMPs showed severely reduced translocation of K^+ and of H^+. Actually, they did not reveal any significant extrusion of H^+. K^+ uptake into *yol027*Δ SMPs was reduced by 95% as compared to wild-type SMPs. The residual activity may reflect K^+ uniport. Remarkably, the expression of the human *LETM1* gene in SMPs of mutant *yol027*Δ yeast cells restored K^+/H^+ exchange, although the sequence identity of these proteins is only 34%. Obviously, yeast Yol027p and human Letm1 are orthologs.

The reduced $\Delta[K^+]$ and $\Delta[H^+]$ of mutant SMPs stayed constant for considerable periods of time. The addition of nigericin restored K^+ and H^+ translocations as seen with wild-type SMPs, both with respect to kinetics of H^+ and K^+ movements as well as final concentrations. This indicated that membranes of *yol027*Δ SMPs did not suffer from K^+ and H^+ leakage and that Yol027p could be fully replaced by the exogenous K^+/H^+ exchanger nigericin. We therefore conclude that the differences in K^+ and H^+ fluxes observed between *yol027*Δ and wild-type SMPs solely reflects the absence of Yol027p in the mutant SMPs and that this integral protein of the inner mitochondrial membrane is an essential component of the mitochondrial K^+/H^+ exchange system.

It remains to be shown if the Yol027 or the Letm1 proteins have exchange activity by themselves or if they are essential co-factors in K^+/H^+ exchange systems involving two or more proteins. We are aware that the presence of only a single predicted transmembrane domain would be most unusual for an exchanger. Importantly, the mitochondrial protein fraction shown to reconstitute K^+/H^+ exchange in liposomes [8,20] is in the MW range of 82 kDa. The predicted size of the yeast Yol027 protein and the mammalian Letm1 protein is 65 kDa and 83.4 kDa, respectively. This is in accordance to the role of the Yol027/Letm1 proteins in K^+/H^+ exchange activity.

Acknowledgements

We thank Gerlinde Wiesenberger, Anton Graschopf (both Vienna) and Monika Schweigel (Berlin) for helpful discussion and Mirjana Iliev for excellent technical assistance.

This work was supported by the Austrian Science Fund (FWF).

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