Biochimica et Biophysica Acta, 1108 (1992) 169–176

BBAMEM 75706

K^+/H^+ exchange in yeast mitochondria: sensitivity to inhibitors, solubilization and reconstitution of the activity in proteoliposomes

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(Received 11 November 1991)
(Revised manuscript received 13 March 1992)

Key words: Potassium ion–proton exchange; Mitochondrion; Proteoliposome; Yeast

The K^+/H^+ exhange activity of the inner mitochondrial membrane was investigated in the yeast *Saccharomyces cerevisiae*. Swelling experiments in potassium acetate indicated that the K^+/H^+ exchange was active without any additional treatment after the mitochondria isolation, such as a Mg^{2+} depletion. As in mammalian mitochondria, the activity of yeast mitochondria was stimulated by increasing pH and was inhibited by the amphiphilic amines quinine and propranolol and by the carboxyl reagent dicyclohexylcarbodiimide. However, the activity was poorly inhibited by Mg^{2+} and consequently was only slightly stimulated by the Mg^{2+}/H^+ exchanger A23187. On the other hand, Zn^{2+} was very efficient for inhibiting the exchange and consequently the activity was strongly stimulated by the permeant metal-chelator *o*-phenanthroline. The [^{86}Rb]Rb^+ accumulation in mitochondria and mitoplasts was only partially inhibited by quinine and propranolol suggesting that part of the accumulation monitored under these conditions was due to cation leak through the inner membrane together with adsorption on the membrane. The DCCD-sensitive activity could be reconstituted from mitochondria and from mitoplasts solubilized with Triton X-100; this activity, measured by [^{86}Rb]Rb^+ accumulation, was quinine- and propranolol-sensitive. A spectrophotometric method, based on the capacity of negatively charged proteoliposomes to swell, was then developed in order to continuously follow the reconstituted activity.

1. Introduction

Since the first proposal of the existence of a K^+/H^+ exchange through the inner mitochondrial membrane [1], this system has been intensively studied in rat-liver mitochondria essentially by the group of Garlid [2–11]. It could be unambiguously differentiated from simple cation leaks since it promoted electroneutral potassium efflux [3]. It was activated by Mg^{2+} depletion [2,5] that allowed the authors to propose a 'Mg^{2+} carrier-brake' model for its regulation [3]. However, other data reported by Bernardi and Azzone [12] supported the view that the A23187-induced K^+/H^+ exchange activity was regulated by the transmembrane potential, independently on the extent of Mg^{2+} depletion.

The K^+/H^+ exchange activity was inhibited by several amphiphilic amines such as quinine and propranolol [4] and also by the hydrophobic carboxyl-reagent dicyclohexylcarbodiimide (DCCD), the effect of which was prevented by Mg^{2+} and quinine [6–8]. This last characteristics allowed the group of Garlid to purify a [^{14}C]DCCD-labeled protein of 82 kDa able to accumulate K^+ and Rb^+ into proteoliposomes, following an electroneutral mode [9–11].

Little is known about this system in yeast mitochondria despite the fact that the molecular approach could provide a good support for investigating its physiological regulation. It has been shown that yeast mitochondria largely swell when suspended in acetate salts [13–15] and that this swelling was inhibited by quinine [15]. It is also well-known that the coupling state of yeast mitochondria is strongly sensitive to the presence of cations (potassium and sodium) in the incubation medium [16]. Results reported by Dabadie et al. [15] suggested that, in this organism, this exchange was more active than in mammalian mitochondria.

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Abbreviations: CCCP, *m*-carbonylcyanide *p*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide.

In this paper, we have investigated some characteristics of the K^+/H^+ exchange of the inner membrane of yeast mitochondria and have checked that most of the properties described in mammalian mitochondria could also be found in yeast mitochondria, with the major exception that the exchange was poorly Mg^{2+}-sensitive. On the other hand, we have found a strong Zn^{2+} sensitivity. We reconstituted the K^+/H^+ exchange activity into proteoliposomes after mitochondria or mitoplasts solubilization with Triton X-100, that brought a support for a further purification of the activity. This reconstituted activity, as the native one, was DCCD-, quinine- and propranolol-sensitive.

2. Materials and Methods

Cells of the haploid strain AB1-4A/8 (mat a, his4) were grown at 28°C in a 1% Yeast Extract, 0.1% potassium dihydrogenophosphate, 0.12% ammonium sulfate medium (pH 4.5) supplemented with 3% glycerol. Cells were harvested in the mid-exponential growth phase and mitochondria were isolated from spheroplasts according to Guérin et al. [17]. Mitochondria were finally suspended at 40 mg/ml in a 0.6 M mannitol, 2 mM Tris-EGTA, 10 mM Tris-maleate buffer (pH 6.8) (Buffer A). Protein content was measured by the biuret method [18] in mitochondria and mitoplasts, and by the amido black method [19] in solubilized fractions and proteoliposomes using bovine serum albumin as standard in both cases. Mitochondria could be frozen as small beads in liquid nitrogen and stored at −80°C without any significant alterations of the properties described below.

Mitochondria swelling was followed as the decrease of the absorbance of mitochondria suspensions at 578 nm in 0.3 M potassium acetate or potassium chloride buffered with 10 mM Tris-maleate (pH 6.8, except when indicated). All the experiments were performed at room temperature, at a mitochondrial concentration of 1 mg/ml. The salt concentration of 0.3 M was chosen because it allowed a full reversibility of the swelling after suspending preswollen mitochondria in a mannitol solution. This full reversibility was not observed when the salt concentration was decreased down to 0.2 M (not shown).

Mg^{2+} and K^+ contents of freshly isolated mitochondria were monitored by atomic absorption spectroscopy after centrifugation of mitochondria through silicon oil and precipitation of mitochondrial proteins by 0.3 M trichloroacetic acid.

Mitoplasts were prepared by digitonin-treatment of mitochondria according to Velours et al. [20] and purified on a 10 ml linear sucrose gradient (65–25% (w/v) adjusted at pH 6.8 with 10 mM Tris-maleate) submitted to a 105000 × g centrifugation for 90 min.

[^{86}Rb]Rb^+ accumulation in mitochondria and mitoplasts was monitored at room temperature by incubating them in 1 ml of buffer A, in the presence of antimycin A (0.2 μg/mg) and oligomycin (5 μg/mg) and 9 mM [^{86}Rb]RbCl (500–700 cpm/nmol). Tubes were then centrifuged for 3 min at 17000 × g and the pellets were dissolved in 0.2 ml formic acid before counting.

Solubilization of mitoplasts and reconstitution of the exchange activity was performed as follows. Mitoplasts (10 mg/ml) were preincubated for 15 min at 4°C in the presence or in the absence of DCCD (50 μg/mg proteins) (since DCCD was dissolved in methanol, control tubes contained an equivalent volume of this solvent). Mitoplasts were then diluted to 2 mg/ml in a 5 mM Tris-EDTA, 10 mM Hepes buffer, pH 7.2 (buffer B), and added with a 10% (w/v) Triton X-100 solution prepared in buffer B (2.5 mg detergent/mg protein). After a 40-min incubation at 4°C, the pellet of insolubilized material was removed by a 35-min centrifugation at 150000 × g.

Phosphatidylcholine was obtained from crude soybean asolectin (Sigma) according to Soper et al. [21]. The phospholipid content was measured according to Guérin and Napias [22].

A mixture of 47 mg of phosphatidylcholine and 0.5 mg of beef heart cardiolipin (Sigma) was dessicated under nitrogen and then evaporated overnight under vacuum to eliminate remaining traces of organic solvents. The lipid mixture was then dispersed in 1 ml of 5 mM Tris-EDTA, 120 mM Hepes, pH 7.4 (Buffer C) and submitted to a 3–5-min sonication on ice (up to clarity of the suspension). 0.45 ml of liposomes (21.4 mg of lipids) was added with various amounts (0.1 to 0.25 mg) of the protein extract (or with the same volume of buffer B added with an equivalent amount of Triton X-100) and the mixture was added to 300 mg of Bio-Beads SM_2 (Bio-Rad) pre-equilibrated for 2 h with 3 ml of a degazed mixture of buffers B and C (1:1), and gently stirred at room temperature for 2 h.

K^+/H^+ exchange activity was monitored with two different methods. (i) Accumulation of [^{86}Rb]Rb^+: aliquots (about 2 mg lipids) of liposomes or proteoliposomes were added to 900 μl buffer C. 9 mM [^{86}Rb]rubidium chloride (500–700 cpm/nmol) were added. Assays were incubated for 3 min at room temperature and the accumulation was stopped by a 15-min centrifugation at 130000 × g at 4°C. Supernatant was removed and the pellet was dissolved in 0.2 ml formic acid before counting. (ii) Under adequate osmotic conditions, the activity could be followed by monitoring the swelling of liposomes or proteoliposomes by the decrease in optical density at 520 nm (see Results).

The internal volume of liposomes was monitored by incubating liposomes or proteoliposomes with [^{3}H]H_2O (45000 cpm/μl) and [^{14}C]mannitol (5000 cpm/μl) for

5 min before a 15-min centrifugation at 130000 × g. The radioactivity was then counted in both pellet and supernatant.

3. Results and Discussion

Swelling of mitochondria suspended in potassium acetate

Mitochondria swelled spontaneously when suspended in a 0.3 M solution of potassium acetate buffered at pH 6.8 (Fig. 1A, trace a). This swelling was not altered by antimycin A or oligomycin addition (not shown) evidencing its strictly non-energetic nature. It was biphasic, the swelling rate increasing after 3 to 5 min, depending on mitochondrial preparations. ClCCP did not inhibit nor stimulate the rate of this swelling (Fig. 1A, trace b).

As in mammalian mitochondria, the swelling in potassium acetate was inhibited by preincubating mitochondria in the presence of the carboxyl reagent DCCD (Fig. 1B). The amphiphilic amines quinine and propranolol also strongly inhibited the swelling (Fig. 2A). The dextrorotary stereoisomer of quinine, quinidine, inhibited the swelling of yeast mitochondria (Fig. 2A) but not that of beef-heart mitochondria [15].

In mammalian mitochondria, the addition of the Mg^{2+}/H^+ exchanger A23187 induced both a reduction of the lag and a strong stimulation of the K^+/H^+ exhange [6]. In yeast mitochondria, addition of A23187 promoted only a slight reduction of the lag before swelling and practically no stimulation of the swelling rate (Fig. 1A, trace c). The matricial Mg^{2+} content varied between 20 and 30 nmol per mg protein in

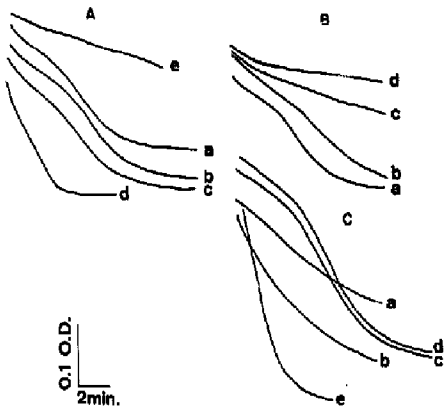

Fig. 1. Non-energetic swelling of mitochondria suspended in isoosmotic solutions of potassium salts. Mitochondria (1 mg/ml in A and B, 2 mg/ml in C) were suspended at room temperature in a 0.3 M potassium acetate, 10 mM Tris-maleate buffer (pH 6.8) (A, B) or 0.3 M potassium chloride, 10 mM Tris-maleate buffer (pH 6.8) (C). The decrease in optical density was followed at 578 nm. (A) Effect of ionophores on the swelling in potassium acetate. Trace a, no addition; trace b, +CCCP 6 μM; trace c, A23187 1 μg/mg; trace d, + o-phenanthroline 2 mM; trace e, mitochondria treated with 50 μg DCCD/mg (see below)+ o-phenanthroline 2 mM. (B) Effect of DCCD on the swelling in potassium acetate. Mitochondria were preincubated at 4°C for half an hour in a small volume (50 μl) added with methanol (5 μl) containing 0 (a), 5 (b), 10 (c) or 50 (d) μg DCCD per mg proteins. (C) Mitochondria were suspended in 0.3 M potassium chloride. Trace a, no addition; trace b, +CCCP 6 μM; trace c, +triethyltin 10 μM; trace e, +valinomycin 250 ng/mg. Trace d is a swelling in potassium acetate measured on the same mitochondria preparation.

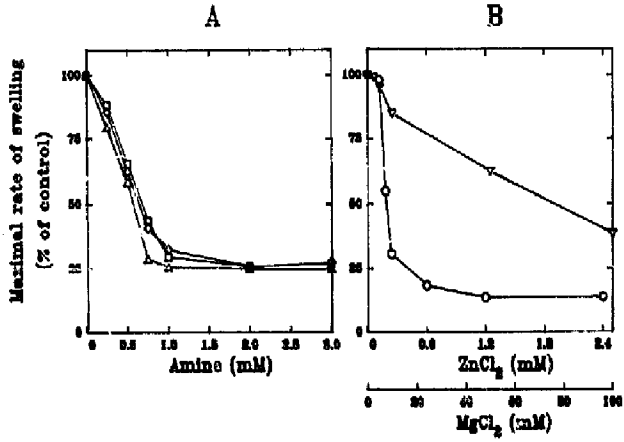

Fig. 2. Effect of the inhibitors on the maximal swelling rate. The maximal swelling rates were calculated from the derivatives of the swelling curves obtained as in Fig. 1. (A) Effect of amphiphilic amines: quinine (□), quinidine (○) and propranolol (△). (B) Effect of divalent cations: (▽) Mg^{2+} and (○) Zn^{2+}.

freshly isolated mitochondria, not significantly different from the values obtained in rat-liver [12], and therefore cannot account for the difference in A23187 sensitivity. It should also be noted that mitochondria isolated with buffers without added EGTA did not exhibit significantly different characteristics as compared to mitochondria isolated in the classical buffers added with EGTA (not shown).

The effect of exogenous Mg^{2+} was then assayed (Fig. 2B). Mg^{2+} inhibited swelling but the effective concentrations were about 50-fold higher than those reported in mammalian mitochondria [12]. High Ca^{2+} concentrations (up to 100 mM $CaCl_2$) had no effect on swelling (not shown), which was different from reports on mammals [5], and was consistent with the assumption that yeast mitochondria fail to support any Ca^{2+} transport pathway [14,23,24].

Since yeast mitochondria also contain free Zn^{2+} [25], we assayed the effect of this cation on the exchange activity. Zn^{2+} was found to strongly inhibit the exchange with a $K_{1/2}$ of 0.2 to 0.3 mM (Fig. 2B), close to the K_i reported for Mg^{2+} in rat-liver mitochondria [12]. Consequently, the effect of the permeant metal-chelator *o*-phenanthroline was assayed (Fig. 1A, trace d); it strongly stimulated the swelling until a maximal rate and therefore had the same effect as that reported for A23187 in mammalian mitochondria. Opposite to the exogenous K^+/H^+ exchanger nigericin (not shown), *o*-phenanthroline did not induce any swelling of mitochondria pretreated with DCCD (Fig. 1A, trace e) that showed that the chelator had no ionophoric effect by itself. The effect that we observed cannot therefore be related to the uncoupling effect of (*o*-phenanthroline)$_2$-Cu^{2+} observed by Shinoara and Terada [26].

We have also investigated whether Zn^{2+} had the same effect on rat liver mitochondria. After depletion of divalent cations by a pretreatment with A23187 and EDTA [2], we observed the same inhibitory effect of Zn^{2+} on the swelling in potassium acetate as for yeast mitochondria (not shown).

A control of the electroneutral nature of the K^+ entry pathway involved in the swelling of mitochondria suspended in potassium acetate was made by comparing this swelling to that obtained in potassium chloride 0.3 M (Fig. 1C, trace a). Mitochondria swelled poorly when suspended in potassium chloride. This swelling was strongly stimulated by CCCP (Fig. 1c, trace b). It was insensitive to quinine, propranolol and DCCD (not shown). Interestingly, the addition of triethyltin (Fig. 1C, trace c) promoted a swelling, the profile of which was strictly the same as that obtained in potassium acetate (Fig. 1C, trace d). According to Selwyn et al. [30], these results would indicate that yeast mitochondria support a poor ability to swell by a mechanism of associated electrogenic transport of K^+ and Cl^- and that the addition of triethyltin would allow a swelling by a mechanism of associated electroneutral exchanges K^+/H^+ (catalyzed by the exchanger) and Cl^-/OH^- (catalyzed by triethyltin). It should be noted that, in the absence of triethyltin, the addition of valinomycin induced a large and rapid swelling (Fig. 1C, trace e) showing that the electrogenic entry of Cl^- was not a limiting factor for the swelling in KCl.

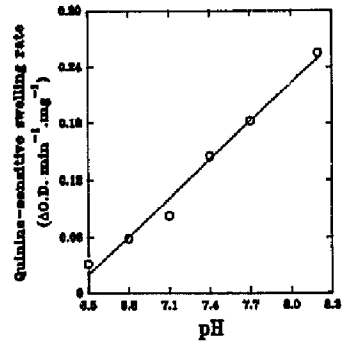

Fig. 3. Effect of pH on the quinine-sensitive swelling in potassium acetate. Each point corresponds to the difference between the maximal swelling rate in the absence and in the presence of 1 mM quinine.

The effect of pH on the K^+/H^+ exchange was investigated by plotting the maximal rate of swelling minus the rate of swelling in the presence of 1 mM quinine versus pH (Fig. 3). As for mammalian mitochondria, the quinine-sensitive swelling was strongly stimulated by increasing pH.

It should be noted that both the *o*-phenanthroline treatment (Fig. 1) and the high pH (not shown) made the lag before the rapid swelling phase disappear. This ressembled to the observations reported on rat liver mitochondria [2–5]. On this material, the lag was interpreted as the time necessary to unmask the exchange activity (by cations depletion and/or increase of the matricial pH [9]); such an interpretation could also be proposed for yeast mitochondria, with the difference that, in isolated yeast mitochondria, removal of physiological mechanisms of inhibition of the exchange should be spontaneous.

Accumulation of [^{86}Rb]Rb^+ into mitochondria and mitoplasts

We measured the accumulation of the K^+ analog Rb^+ into mitochondria and mitoplasts. preliminary experiments showed that the characteristics of the swelling of mitochondria suspended in rubidium acetate were the same as in potassium acetate although less rapid (not shown).

Non-energized mitochondria (+antimycin A and oligomycin) accumulated Rb^+ but only 37% of the

total accumulation was sensitive to propranolol (Table I). A possibility was that most of the accumulation was due to contaminating vesicles; consequently, mitochondria were purified on a sucrose gradient: the ratio of propranolol-sensitive accumulation was not increased (not shown). Similar results were obtained with mitoplasts showing that the inhibitor-insensitive accumulation was not due to contaminating vesicles nor to the intermembrane space. This phenomenon appeared to be very rapid since it occurred within 15 s (see Fig. 4).

The rate of propranolol-sensitive Rb^+ accumulation in mitochondria appeared to correspond to the rate of swelling in potassium acetate since the maximal accumulation was reached after 7 to 10 min (Fig. 4).

In mitochondria, the accumulation of $[^{86}Rb]Rb^+$ that we measured could correspond to the sum of both a $[^{86}Rb]Rb^+/H^+$ exchange and a $[^{86}Rb]Rb^+/K^+$ exchange since the matrix contained about 100 nmoles K^+ per mg protein (determined by atomic absorption spectroscopy). This observation could explain an apparent contradiction between swelling experiments and $[^{86}Rb]Rb^+$ accumulation experiments. The fact that no lag was observed before to the beginning of propranolol-sensitive accumulation could indicate that the $[^{86}Rb]Rb^+/K^+$ exchange was spontaneous and did not need any removal of a natural physiological mechanism of inhibition, opposite to the K^+/H^+ (or Rb^+/H^+) exchange monitored in swelling experiments.

TABLE I

$[^{86}Rb]Rb^+$ accumulation in mitochondria and mitoplasts

Whole mitochondria (0.5 mg/ml) or mitoplasts (0.28 mg/ml) were suspended at room temperature in a 0.6 M mannitol, 2 mM Tris-EGTA, 0.3% bovine serum albumin, 10 mM Tris-maleate buffer (pH 6.8) containing antimycin A (0.2 μg/mg), oligomycin (5 μg/mg) and $[^{86}Rb]RbCl$ (9 mM, 500–700 cpm/nmol). After a 5-min incubation at room temperature, tubes were rapidly centrifuged at 17000 × g, the supernatant was removed and the pellet was dissolved in 0.2 ml formic acid before counting. Results presented in the table were obtained on the same mitochondria preparation (n = number of independent determinations; results are means ± S.D.). Similar results were obtained with three different mitochondria preparations).

	$[^{86}Rb]Rb$ accumulation (nmol Rb^+ per mg)
Mitochondria	
Control	190 ± 30 (n = 11)
+ propranolol 1 mM	120 ± 15 (n = 11)
+ quinine 1 mM	134 ± 11 (n = 4)
+ CCCP 4 μM	174 ± 15 (n = 4)
+ CCCP 4 μM + quinine 1 mM	92 ± 19 (n = 4)
Mitoplasts	
Control	155 ± 3 (n = 3)
+ quinine 1 mM	111 ± 6 (n = 3)
+ CCCP 4 μM	177 ± 5 (n = 3)
+ CCCP 4 μM + quinine 1 mM	94 ± 6 (n = 3)

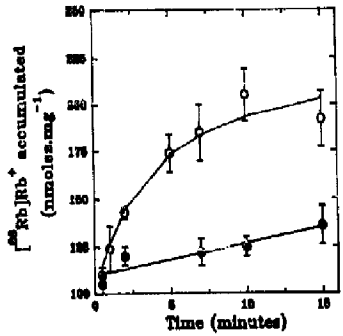

Fig. 4. Time-dependence of the $[^{86}Rb]Rb^+$ accumulation in mitochondria. Mitochondria (0.5 mg/ml) were suspended at room temperature in a 0.6 M mannitol, 2 mM EGTA, 0.3% bovine serum albumin, 10 mM Tris-maleate buffer (pH 6.8) added with antimycin A (0.2 μg/mg), oligomycin (5 μg/mg) in the absence (○) or in the presence (●) of propranolol (1 mM). At t = 0, 9 mM $[^{86}Rb]RbCl$ (200 cpm/nmol) were added and the incubation was stopped after the indicated time by a rapid centrifugation. The supernatant was removed and the pellet was dissolved in 0.2 ml formic acid before counting.

The fact that the accumulation of $[^{86}Rb]Rb^+$ accumulation in whole mitochondria essentially involved a $[^{86}Rb]Rb^+/K^+$ exchange was supported by the fact that CCCP had no clear effects on this accumulation (Table I). On the other hand, CCCP significantly stimulated the accumulation in mitoplasts showing that, under these conditions, we essentially measured the activity of the $[^{86}Rb]Rb^+/H^+$ exchange.

However, although significant, the increase induced by CCCP on $[^{86}Rb]Rb^+$ accumulation in mitoplasts is not very large, suggesting that a part of $[^{86}Rb]Rb^+$ accumulation is due to electrophoretic pathways, probably leaks. This hypothesis was supported by the fact that, in the presence of quinine, CCCP inhibited the residual $[^{86}Rb]Rb^+$ accumulation (Table I). The remaining $[^{86}Rb]Rb^+$ value obtained in the presence of quinine and CCCP could probably account for the adsorption of the cation on the membrane.

The difference between mitochondria and mitoplasts, as regards the effect of CCCP, could be due to the loss of matricial K^+ during the centrifugation through the sucrose gradient like for most of matricial metabolites and proteins, as it could be observed on SDS-PAGE (not shown).

Solubilization and reconstitution of the exchange activity into proteoliposomes

A method was developed to reconstitute the activity from solubilized membrane proteins, in order to further follow the activity of purified fractions.

In a first set of experiments, whole mitochondria were solubilized with different Triton X-100 concentra-

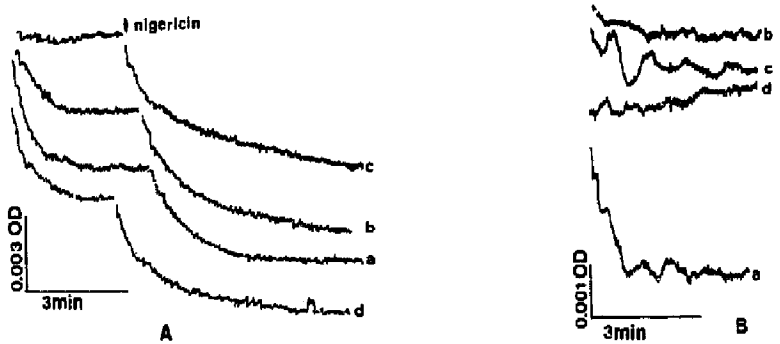

Fig. 5. Swelling of liposomes and proteoliposomes in isoosmotic solutions of potassium acetate. Liposomes and proteoliposomes were suspended in a 60 mM potassium acetate solution adjusted at pH 7.3 (A) or 6.5 (B) with Tris-HCl. The decrease in optical density was followed at 520 nm. In (A), the concentration of liposomes and proteoliposomes was 3 mg lipids/ml, and this concentration was 1.6 mg/ml in (B). In both cases, the lipid/protein ratio was 140 (w/w). Traces a, proteoliposomes; traces b, liposomes; traces c, proteoliposomes made with proteins solubilized from mitoplasts preincubated with DCCD (10 μg/mg proteins); traces d, proteoliposomes + quinine 0.5 mM. Nigericin was added at 25 ng/ml.

tions (not shown) which allowed to determine that the activity could be extracted with 2.5 mg Triton X-100/mg protein. Increasing the Triton X-100 amount up to 5 mg/mg protein did not change the activity.

Data in Table 2 report the results of [^{86}Rb]Rb^+ accumulation for two typical experiments: experiment 1 was done with proteins extracted from whole mitochondria and experiment 2 was done with proteins extracted from mitoplasts, in order to avoid any contamination by DCCD-sensitive outer-membrane cations channel [27,28].

TABLE II

Accumulation of [^{86}Rb]Rb^+ in liposomes and proteoliposomes

Liposomes and proteoliposomes (2 mg lipids/ml) were suspended at room temperature in buffer C added with 9 mM [^{86}Rb]rubidium chloride (1000 cpm/nmol) in the presence or in the absence of 1 mM quinine. After a 3-min incubation, tubes were centrifuged for 15 min at 130000× *g*, the supernatant was eliminated and the pellet was dissolved in 0.2 ml formic acid before counting. In experiment 1, the proteins were solubilized from mitochondria. In experiment 2, they were obtained from mitoplasts. The indication '+DCCD' refers to the preincubation of mitochondria or mitoplasts, before the solubilization with Triton X-100. The values are means of four determinations (±S.D.). Similar results were obtained for three independent sets of experiments for extracts from mitochondria and two for extracts from mitoplasts.

	[^{86}Rb]Rb accumulation (nmol Rb^+ per mg lipids)		
	liposomes	proteoliposomes	
		−DCCD	+DCCD
Experiment 1			
Control	–	1.67±0.09	0.77±0.02
+quinine	–	0.82±0.10	0.90±0.02
Experiment 2			
Control	2.80±0.01	3.30±1.00	1.42±0.27
+quinine	2.70±0.09	1.14±0.23	1.53±0.48

In both cases, a large quinine-sensitive accumulation could be monitored in proteoliposomes made with proteins extracted from untreated mitochondria or mitoplasts. When the proteins were solubilized from DCCD-treated mitochondria or mitoplasts, the accumulation was about the same as in control proteoliposomes in the presence of quinine.

It should be noted that a large quinine-insensitive accumulation could be measured in liposomes: that could be due to their large volumes as compared to proteoliposomes (see below), and therefore should correspond to leaks.

We developed another method for continuously following the activity spectrophotometrically, based on the recent observation by Nikaido et al. [29] that the activity of a bacterial porin reconstituted into proteoliposomes could be monitored by the swelling of the proteoliposomes induced by stachyose influx.

In the experiments reported below, the solubilized proteins always originated from mitoplasts. These proteins represented about 40% of the mitoplast proteins and the lipid/protein ratio for the reconstitution was 100 to 200 (w/w).

A first control was performed in order to see whether the volumes of liposomes and proteoliposomes changed or not when suspended in Hepes or potassium acetate solutions (Table III). The volume of liposomes suspended in a 57 mM potassium acetate buffer was about 10% larger than when suspended in a 114 mM Hepes buffer. On the other hand, the volume of proteoliposomes suspended in 57 mM potassium acetate was about 35% larger than when suspended in 114 mM Hepes. From these data, potassium acetate could be

TABLE III

Volumes of liposomes and proteoliposomes before and after swelling

Liposomes or proteoliposomes (2.35 mg lipids/ml) were suspended for 5 min either in a Hepes 114 mM, mannitol 6 mM, pH 6.8 buffer, either in a potassium acetate 57 mM, mannitol 6 mM, pH 6.8 buffer added with [^{3}H]H_2O (45000 cpm/μl) and [^{14}C]mannitol (5000 cpm/μl). After a centrifugation, the radioactivity was counted both in the supernatant and the pellet. Values are means (±S.D.) of four independent determinations.

	Volume (μl per mg lipid)	
	liposomes	proteoliposomes
Hepes buffer	1.44±0.04	0.78±0.19
Potassium acetate buffer	1.58±0.12	1.05±0.26

accumulated in proteoliposomes and this accumulation induced an increase of their volumes.

At pH 7.3, proteoliposomes made with proteins extracted from mitoplasts preincubated in the absence of DCCD swelled rapidly and to a large extent (Fig. 5A, trace a). A further addition of nigericin promoted an additional swelling. A control was obtained with proteoliposomes made with proteins extracted from mitoplasts pretreated with DCCD (Fig. 5A, trace c): these proteoliposomes did not swell spontaneously but the addition of nigericin promoted a large swelling. It should be noted that liposomes, suspended under the same conditions (Fig. 5A, trace b), exhibited a larger swelling than proteoliposomes made with DCCD-treated mitoplasts, which should correspond to the reported above observation (Table II) that they were somewhat leaky.

At such a pH, the inhibitory effect of quinine on proteoliposomes was not always reproducible (Fig. 5A, trace d). Consequently, we tried to find conditions under which the effect of quinine (and/or propranolol) can be clearly observed.

Since the experiments reported by the group of Garlid on the reconstituted activity were performed at pH 8.2 [10], experiments were carried out at this last pH. Essentially similar results were obtained at pH 8.2 and 7.3 (not shown).

On the other hand, decreasing the pH to 6.5 (Fig. 5B) allowed to visualize a clear and reproducible inhibitory effect of quinine (Fig. 5B, trace d) and of propranolol (not shown).

From these data, it appeared that the DCCD-sensitive and quinine- (and propranolol-) sensitive K^+/H^+ exhange activity could be reconstituted from a Triton X-100 extract of yeast mitoplasts.

Conclusion

As mammalian mitochondria, yeast mitochondria supported an active K^+/H^+ exchange. This system appeared to be more active than in mammalian mitochondria since no additionnal treatment (such as Mg^{2+} depletion or preincubation at basic pH) was necessary to evidence it. However, it needed a lag-time before to be activated suggesting that, as in mammalian mitochondria, an intrinsic mechanism of inhibition existed. However, in yeast mitochondria, this mechanism seemed to be spontaneously removed.

Although this exchange presented strong similarities with the system described in mammalian mitochondria (quinine-, propranolol- and DCCD-sensitivity and pH dependence), some differences appeared between both organisms: the K^+/H^+ exchange of yeast mitochondria was quinidine-sensitive and was poorly inhibited by exogenous Mg^{2+} and insensitive to $Ca2+$, possibly because of a different permeability of the inner membrane to these cations as compared to mammalian mitochondria. However, it was strongly inhibited by Zn^{2+} (which was also a K^+/H^+ exchange inhibitor in rat liver mitochondria). Consequently, the swelling was poorly stimulated by the Mg^{2+}/H^+ exchanger A23187, insensitive to the non-permeant chelators EGTA and EDTA, but strongly stimulated by the permeant chelator *o*-phenanthroline.

A Triton X-100 extract from mitochondria was able to induce a Rb^+ accumulation in proteoliposomes. This accumulation was clearly DCCD- and quinine-sensitive. The activity could also be followed by the swelling of proteoliposomes in potassium acetate, which was DCCD-, quinine- and propranolol-sensitive. These characteristics allowed us to conclude that the K^+/H^+ exchange activity of the inner membrane of yeast mitochondria could be solubilized and reconstituted with essentially similar properties as the native activity. The purification of the protein involved in this K^+/H^+ exchange is underway.

Acknowledgements

The authors thank Prof. André Lamant for atomic absorption spectroscopy measurements, Muriel Jumelle-Laclau for the kind gift of rat liver mitochondria and Marie-Lise Grellety for language correction. This work was supported by grants from the Université de Bordeaux II, the Centre National de la Recherche Scientifique and the Conseil Régional d'Aquitaine.

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