Functional characterization of the partially purified Sac1p independent adenine nucleotide transport system (ANTS) from yeast endoplasmic reticulum

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Several ATP-depending reactions take place in the endoplasmic reticulum (ER). Although in Saccharomyces cerevisiae ER the existence of a Sac1p-dependent ATP transport system was already known, its direct involvement in ATP transport was excluded. Here we report an extensive biochemical characterization of a partially purified adenine nucleotide transport system (ANTS) not dependent on Sac1p. Highly purified ER membranes from the wild-type and Δsac1 yeast strains reconstituted into liposomes transported ATP with the same efficiency. A chromatography on hydroxyapatite was used to partially purify ANTS from Δsac1 ER extract. The two ANTS-enriched transport activity eluted fractions showed essentially the presence of four bands, one having an apparent MW of 56 kDa, similar to that observed for ANTS identified in rat liver ER. The two fractions reconstituted into liposomes efficiently transported, by a strict counter-exchange mechanism, ATP and ADP. ATP transport was saturable with a Km of 0.28 mM. The ATP/ADP exchange mechanism and the kinetic constants suggest that the main physiological role of ANTS is to catalyse the transport of ATP into ER, where it is used in several energy-requiring reactions and to export back to the cytosol the ADP produced.

The endoplasmic reticulum (ER) is a membrane network (1) found in every nucleated cell. The morphologically distinct smooth and rough ER (SER and RER, respectively) are formed by the same continuous membrane as the nuclear envelope. Its internal compartment, the ER lumen, is completely separated from the cytosol. This compartmentation often narrows the specificity of luminal enzymes (2) because several potential substrates cannot pass the barrier. The transport of selected substrates across the ER membrane is an additional point where enzyme activity can be potentially regulated. It is, therefore, doubtless that ER functions cannot be properly revealed without understanding the related transport processes which, in turn, require the characterization and identification of the participating membrane proteins.

The lumens of the ER and Golgi apparatus are the subcellular sites where dissociation of chaperones-proteins complexes, disulphide bridge formation, protein polymerization, glycosylation and phosphorylation of proteins, proteoglycans or lipids occur (3–9). It is known that ATP is utilized in the RER lumen in all reactions listed above as energy source (2). Furthermore, the degradation of misfolded or overexpressed proteins, a process that requires ATP, has been postulated to occur within the ER lumen (10, 11). ATP is synthesized in cytosol during glycolysis and oxidative phosphorylation within the mitochondria, where is exchanged with the cytosolic ADP by the action of the mitochondrial ADP/ATP carrier (AAC) (12, 13).
Cytoplasmic ATP is imported by a different transport system in various organelles such as peroxisomes (14, 15) and ER (16–18).

The existence of an ATP transport system inhibited by 4,4′-disothiocyanato-2,2′-stilbenedisulfonic acid (DIDS) was demonstrated in ER-derived vesicles prepared from rat liver and canine pancreas (16).

In rat liver ER a saturable transport, highly specific for ATP, was measured in a reconstituted system (19), successively a 56-kDa protein was identified as an ATP transporter by photoaffinity labelling and partial purification (20). In a more recent study (21) an ATP transporter from rat liver RER was solubilized and reconstituted into phosphatidylcholine liposomes, ATP transport was time and temperature dependent, and inhibited by DIDS, but it was unaffected by carbboxyatractylsido, a specific inhibitor of the mitochondrial AAC (13, 22).

An ATP transport system and its inhibition by DIDS was also demonstrated in Saccharomyces cerevisiae ER (17). In yeast ER a 68-kDa protein (Sac1p) was identified as a carrier responsible for ATP transport during protein translocation (23). However, it turned out that Sac1p was not an ATP transporter itself, since purified Sac1p reconstituted into proteoliposomes did not catalyse any ATP uptake (24), rather it acted as an important regulator of the transport process, probably by controlling ER phosphoinositides. The absence of Sac1p resulted in a defect in microsomal ATP transport; however, microsomes from sac1Δ strain retained 15% of ATP transport rate suggesting that another ATP transporter Sac1p independent was present in the microsomal membranes (23).

Here we report the biochemical characterization and the partial purification of a Sac1p independent adenine nucleotide transport system (ANTS) in yeast ER.

Materials and Methods

Yeast strains and growth

BY4742 (wild-type), sac1Δ (YKL212W), aac1Δ (YMR056C), aac3Δ (YBR085W), mne1Δ (YMR166C), ymc2Δ (YBR104W), ndl2Δ (YEL066W), lee1Δ (YHR020W), mrs1Δ (YJL133W), mrs3Δ (YKR052C), mnr1Δ (YGR257C), flx1Δ (YIL134W), YPR038WΔ and anti1Δ (YPR128C) yeast strains were provided by the EUROFAN resource Centre EUROSCARF (Frankfurt, Germany). Yeast strains and growth conditions are described in Materials and Methods.

Isolation of yeast organelles

Mitochondria/peroxisomes and microsomes were isolated from the wild-type and deletion strains grown in YPD until the early exponential phase (optical density between 1.0 and 1.5) was reached. The cells were pelleted by centrifugation at 3,000 × g for 5 min at room temperature and washed with distilled water, then they were suspended in 2 ml/g cells (w/v) DETE buffer (100 mM Tris–HCl, pH 9.4, 10 mM MgCl2, 4–dithioerythritol) and shaken slowly for 10 min at 30°C. The pellet was washed with zymolyase buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) and then incubated in 7 ml/g of zymolyase buffer with the addition of 4 mg/g (w/v) Zymolyase-20T (Seikagaku Kogyo Co.) for 45 min at 30°C for conversion into spheroplasts. Homogenization was carried out by 30 strokes in a glass–Teflon potter in 14 ml/g (w/v) ice-cold homogenization buffer (0.6 M sorbitol, 10 mM Tris–HCl, pH 7.4, 1 mM PMSEF, 0.2% BSA). This homogenate was diluted with 1 volume of ice-cold homogenization buffer, then cell debris and nuclei were removed by centrifugation at 3,000 × g for 5 min at 4°C. The supernatant was centrifuged twice at 12,000 × g for 15 min at 4°C to recover the mitochondrial/peroxisomal fraction, which was suspended in ST buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.0) to a final concentration of 10 mg of protein/ml. The supernatant was centrifuged at 100,000 × g for 60 min at 4°C, and the resulting microsomal fraction was suspended in ST buffer to a final concentration of 10 mg of protein/ml. About 1 or 10 mg of mitochondrial/peroxisomal and microsomal fractions were stored at −80°C as pellet until use.

Partial purification of ANTS

About 10 mg of frozen microsomes were suspended at a final concentration of 10 mg protein/ml in a solubilization buffer containing 2.5% Triton X-100, 30 mM NaCl, 10 mM PIPES pH 7 and 10 mg/ml asolectin. After 30 min at 4°C the mixture was centrifuged at 100,000 × g for 30 min to obtain a clear supernatant referred to as extract, then 800 μl of extract (5–6 mg protein) were applied on cold hydroxyapatite (HTP) columns (a Pasteur pipette containing 600 mg of dry material) and the elution was performed using the solubilization buffer. Two consecutive fractions, each of 600 μl were collected, 100 μl of each fraction were reconstituted into liposomes.

Reconstitution of ANTS

When microsomal membrane extracts were reconstituted, 1 mg of frozen microsomes was solubilized for 30 min at 4°C in 1 ml of a solubilization buffer containing 2.5% Triton X-100, 30 mM NaCl, and 10 mM PIPES pH 7 and centrifuged for 30 min at 100,000 × g. The microsomal extracts and the HTP eluates were reconstituted into liposomes by the cyclic detergent removal procedure (25).

In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through a column filled with Bio-Beads SM-2 resin (Bio-Rad Milan, Italy) to remove the detergent. The composition of the initial mixture used for reconstitution was: 100 μl of extracts or HTP eluates (about 80–100 μg and 70–90 ng of proteins, respectively), 70 μl of 10% Triton X-114 (TX-114), 100 μl of liposomes (10 mg of L-α-phosphatidylcholine from egg yolk), prepared as described previously (26), 2.4 mg of asolectin, 20 mM PIPES pH 7.0 and, when present, 10 mM internal substrate, in a final volume of 700 μl. After vortexing, this mixture was passed 13 times through the same Bio-Beads SM-2 column (0.5 × 3.5 cm) equilibrated with a buffer containing 10 mM PIPES pH 7.0.

All the operations were performed at 4°C, except the passages through Bio-Beads SM-2 column that were performed at room temperature (27).

Transport measurements

Approximately 600 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 × 15 cm) pre-equilibrated with 50 mM NaCl/10 mM PIPES/NaOH pH 7.0 (buffer A). The first 600 μl of the flow through eluate from the Sephadex column were collected, transferred to reaction vessels (100 μl each), and used for transport measurements by the inhibitor stop method (28, 29). Transport was started by adding external labelled substrate at the concentrations indicated in the figure legends. After the required time interval, the reaction was stopped by adding 80 mM pyridoxal 5'-phosphate (PLP), in control samples, the inhibitor was added together with the labelled substrate at time zero. The assay temperature was 25°C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6 × 13 cm) to separate the external from the internal radioactivity. The liposomes eluted with 3 ml of 50 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted. For the determination of the [14C]ATP uptake, the experimental values were corrected by subtracting the respective controls (samples inhibited at time zero). For kinetic determinations, the initial transport rate was evaluated from the radioactivity taken up by the proteoliposomes in 1 min, i.e. within the linear range of the substrate uptake. Specific transport was referred to the amount of protein in the proteoliposomes (30–32). For efflux measurements, proteoliposomes containing 2 mM ATP were labelled with 0.01 μM [3H]ATP by carrier-mediated exchange equilibration for 40 min, external radioactivity was removed by passing the proteoliposomes through Sephadex G-75 column. Efflux was initiated by adding unlabelled external substrate or buffer A alone and terminated by the addition of the inhibitor above indicated.
Mass spectrometry and gel digestion
In gel digestion: isolate protein band was destained with a freshly prepared destaining solution (20mL) that contained 30mM potassium ferricyanide and 50mM sodium thiosulfate. The destaining solution was removed, and the gel pieces were washed with 50mL water (5–7 times) until the yellow colour disappeared. The gel pieces were washed (two times) with 50μL of 50% acetonitrile (ACN): 50% 200mM ammonium bicarbonate for 5 min, dehydrated with 50μL of 100% ACN until the gel turned opaque white and dried in a vacuum centrifuge for 30 min. The gel pieces were rehydrated in 5–10μL of 50mM ammonium bicarbonate (37°C, 4 min), an equivalent volume (2–5μL) of trypsin (Sigma Aldrich) solution (4pmol/μL) was added. After digestion peptides were recovered by sonication (10 min) in 50μL of solution, 60% ACN in 5% TFA (vol/vol). The supernatants were pooled and dried by Concentrator Plus system.

Mass spectrometer: MALDI MS and MS/MS analysis. Linear MALDI-TOF spectra were acquired using a 5800 MALDI-TOF/TOF analyser (AB SCIEX, Darmstadt, Germany) equipped with a neodymium: yttrium-aluminium-garnet laser (laser wavelength: 349 nm). About 1μL portion of a premixed solution of sample and α-CHCA (0.3% in TFA) was spotted on the matrix target, dried at room temperature and analysed in the mass spectrometer. Spectra were acquired in default calibration mode averaging 2,500 laser shots with a mass accuracy of 50 ppm. Besides, MS and MS/MS analyses were performed with a 5800 MALDI-TOF/TOF analyser in reflectron positive-ion mode. About 1 μL of sample matrix mixed solution was spotted on a MALDI plate and dried. MS spectra were acquired with a laser pulse rate of 400 Hz and at least 4,000 laser shots, while CID-MS/MS experiments were performed at a constant energy of 35 eV, using ambient air at collision gas with a medium pressure of 10⁻⁸ Torr. CID-MS/MS spectra required up to 5,000 laser shots and a pulse rate of 1,000 Hz.

Database searching and bioinformatics. Proteins were identified by searching a comprehensive protein database using Mascot programs (www.matrixscience.com). Peak harvesting was done automatically using Data Explorer software (Applied Biosystems). Peak resolution parameters able to affect the percentage of the active transporter extracted by the detergent from the microsomal membranes and the efficiency of its incorporation into liposomes, i.e. the detergent/lipid ratio, the number of passages through the same Bio-Beads SM-2 column and the pH of the reconstitution mixture (38).

Others methods
Proteins were analysed by SDS-PAGE (35) and stained with Coomassie Blue dye or staining (36). The amount of protein incorporated into liposomes was measured as described previously (37, 38). Western blot analysis was carried out as described formerly (29–41) using an anti-complex IV subunit III (anti-COX) (Invitrogen, Milan, Italy) and an anti-dolichol phosphate mannose synthase (DPM1) antibodies (Invitrogen, Milan, Italy), specific for the mitochondrial and microsomal fractions, respectively. The antibody against yeast Auc1p was kindly provided by Pelosi (42).

Results
Optimization of the solubilization procedure
In order to functionally characterize and to identify the microsomal ANTS, we purified the mitochondrial/peroxisomal and microsomal fractions from wild-type and sac1Δ yeast strains by differential centrifugation from yeast homogenate; the latter was used to unravel the role of Sac1p in the transport of ATP across the ER membrane. The two fractions were chromatographed on SDS-PAGE and tested for their purity by western blot analysis. The yeast anti-COX1 antibody immunodecorated a single band with an apparent molecular mass of about 26kDa only in the mitochondrial/peroxisomal fractions (Fig. 1A, lane M/P); whereas the yeast anti-DPM1 antibody immunodecorated a single band with an apparent molecular mass of about 30kDa only in microsomal fractions (Fig. 1A, lane ER).

Initially the transport measurements were carried out on microsomal fractions purified from both wild-type and sac1Δ yeast strains. No difference was found between the two fractions concerning their efficiency to transport ATP and their sensitivity to the inhibitors DIDS and carboxyatractyloside, in all the initial reconstitution conditions tested (Fig. 1B). Once excluded a possible involvement of Sac1p in the ER ATP transport, the remaining transport experiments were carried out only on the microsomal fraction purified from sac1Δ yeast strain.

In order to better understand the mechanism of functioning of this transport system, we first optimized the reconstitution procedure of the microsomal fraction. This aim was reached by adjusting the main parameters able to affect the percentage of the active transporter extracted by the detergent from the microsomal membranes and the efficiency of its incorporation into liposomes, i.e. the detergent/lipid ratio, the number of passages through the same Bio-Beads SM-2 column and the pH of the reconstitution mixture (38). The solubilization of membrane proteins by detergents is a critical step to obtain active extracted proteins suitable for the further successful biochemical characterization (43). The same amount of microsomal membranes (about 1 mg) was solubilized for 20 min at 4°C with a buffer containing TX-100 or TX-114, two non-ionic detergents, 30mM NaCl and 10mM PIPES, pH 7. On the basis of our previous experience on membrane proteins solubilization, two different detergent concentrations were chosen, 1, 5 and 2, 5% (w/v) (38, 43). Once removed the residual unsolubilized material by centrifugation, the supernatant (80–100 μg of proteins) was incorporated into liposomes. In all tested conditions, no significant difference was observed in the measured transport activities, even though the highest activity was found in extracts solubilized in the presence of TX-100 (2, 5% w/v) (Fig. 2A). In order to verify the efficacy of our solubilization procedure in term of amount of transport activity recovered per μg of microsomal membrane proteins used, we solubilized the same amount of ER membranes using 3% TX-100, 150mM Na2SO4 and 10mM Tris–HCl pH 7, as described previously by Mayinger et al. (23); in four independent experiments only 20% of the ATP/ATP exchange activity reported in Fig. 1 was found (data not shown). Several detergent/phospholipid ratios were tested in order to optimize the reconstitution procedure. In such experiments, the [14C]ATP/ATP exchange rate was measured, and the optimal transport activity was obtained by reconstituting the microsomal extracts in the presence of 7 mg of Triton X-114 (1% w/v), 10 mg of 1-α-phosphatidylcholine (1, 42% w/v) and 2, 4 mg of
asolectin (0, 34% w/v), with an optimal detergent/phospholipid ratio of 0.56 w/w (data not shown). Asolectin was required in the reconstitution mixture to get a functional active transporter. The addition in the reconstitution mixture of cardiolipin (20 µg/ml), which was essential to reconstitute in a more active form the mitochondrial AAC carrier (44, 45) (Fig. 2B), or of cholesterol (5 mg/ml), was used to assay the ER Sac1p-dependent ATP transport (23) did not exert any ameliorative effect on the ATP/ATP exchange activity (Fig. 2B). The influence of different pH values in the solubilization buffer and reconstitution mixture on the [14C]ATP/ATP exchange rate was also investigated. The highest transport rates were measured in a range of pH between 7 and 8, even though at pH 7 a higher reproducibility of the maximal transport activity was observed. At pH values lower than 7, the rate of ATP transport drastically decreased (Fig. 2C).

Partial purification of the microsomal ANTS on HTP

In order to identify the putative protein responsible for the [14C]ATP/ATP transport activity measured in the reconstituted microsomal extract, the TX-100 extract of ER membranes was applied on a HTP column and several fractions were recovered by eluting the column with the same solubilization buffer. Only in the first 2 fractions some bands were visible on silver-staining.
SDS-PAGE (Fig. 3A, lanes 3 and 4), 100 μl of each fraction were reconstituted into liposomes. Both reconstituted fractions catalysed an efficient [14C]ATP/ATP exchange reaction (Fig. 3B). By comparing the [14C]ATP/ATP exchange activity catalysed by the reconstituted extract (Fig. 3A, lanes 2) with those of the two fractions (Fig. 3A, lanes 3 and 4), it was evident that the purification step increased ANTS-specific activity by about 950-fold (Fig. 3B). Interestingly, the two HTP fractions showed essentially four bands with apparent molecular weights of 30, 56, 64 and 65 kDa, respectively. Since many members of the mitochondrial carrier family (MCF) have a molecular weight of about 30 kDa, in order to exclude that the 30 kDa band could derive from the presence of one of the functionally unknown or biochemically not characterized mitochondrial carrier (MC) in the ER membrane, we reconstituted the ER membrane extracts of the following deletion strains: \( \text{aac}1 \Delta \) (YMR056C), \( \text{aac}2 \Delta \) (YBR085W), \( \text{mrs}4 \Delta \) (YBR085W), \( \text{mrs}3 \Delta \) (YJR002W), \( \text{mrs}4 \Delta \) (YJR002W), \( \text{mrs}4 \Delta \) (YJR002W), \( \text{yac}1 \Delta \) (YGR257C), \( \text{flx}1 \Delta \) (YIL134W), \( \text{frx}45 \Delta \) and \( \text{ant}1 \Delta \) (YPR128C). The last strain was tested since Ant1p, although biochemically characterized (15), was not only the member of the yeast MCF not localized in the mitochondria but in the peroxisomal membrane. No significant difference was found between the [14C]ATP/ATP exchange reaction catalysed by the \( \text{ace}1 \Delta \) ER extract and those of the various MCs deletion yeast strains (Fig. 3C). Furthermore, we excluded that the 30 kDa band could represent the yeast Aac2p, the most abundant isofrom of the yeast mitochondrial adenine nucleotide carrier, since it did not react against a specific antibody (42). (data not shown). The tryptic digest from 56 kDa gel band was examined in MS reflection mode, whereas MS/MS experiments were used for peptide sequence discrimination. Identification of the peptides, however, was unsuccessful probably due low sample amounts (Supplementary Data).

**Functional characterization of the reconstituted ANTS**

The biochemical characterization of ANTS was carried out on the HTP eluates. Since the patterns of proteins eluted in the two HTP fractions (Fig. 3A, lanes 3 and 4) were similar and the highest [14C]ATP/ATP exchange activity was found not only in the first fraction (Fig. 3, lane 3), but, sometimes, also in the second fraction (Fig. 3, lane 4), we decided to pool the first and second fraction before proceeding to the reconstitution step. The [14C]ATP/ATP exchange activity catalysed by the reconstituted ANTS was fully inhibited by PLP, thus all the transport measurements were carried out by using PLP as stop inhibitor (28). No transport activity was detected when phosphate, glucose, carnitine and glutamine homoexchanges (internal concentration, 10 mM; external concentration 0.1 mM) were checked or with HTP eluates boiled before incorporation into liposomes (data not shown).

ANTS reconstituted into liposomes catalysed a counter-exchange of 0.25 mM external [14C]ATP for 10 μM internal ATP following a first-order kinetic behaviour (rate constant, 0.22 min\(^{-1}\)), isotopic equilibrium being approached exponentially (Fig. 4A). Maximum uptake of [14C]ATP was approached after 20 min. The corresponding value at infinite time was 8.26 μmol/mg protein. The initial rate of ATP uptake (the product of \( k \) and the maximal amount of ATP taken up at equilibrium) was 1.87 μmol/min × mg protein. In contrast, no [14C]ATP uptake was observed without internal substrate (i.e. when, internal 10 mM ATP was replaced with the same internal amount of NaCl), demonstrating that the transporter did not catalyse a unidirectional transport (uniport) (Fig. 4A). This issue was further investigated by measuring the efflux of [14C]ATP from pre-labelled active proteoliposomes, as this provides a more sensitive assay for unidirectional transport (46). In the absence of external substrate, no significant efflux was observed,
even after an incubation of 90 min (Fig. 4B). However, upon addition of external ATP, an extensive efflux of radioactivity occurred. These results showed that the reconstituted ANTS catalysed an obligatory exchange reaction of substrates.

The substrate specificity of ANTS was examined in detail by measuring the rate of [14C]ATP uptake into proteoliposomes that had been pre-loaded with a variety of substrates (Fig. 5A). The highest activities were observed in the presence of internal ATP and ADP. The external [14C]ATP was also exchanged at lower but significant levels with AMP, dATP, dADP, dAMP, GTP, GDP and GMP. Very low transport rates were measured using internal dGTP, dGDP, dGMP, ITP and IDP. No significant exchanges were measured with all the other internal nucleotides tested (Fig. 5A). These results clearly demonstrate that, from the internal side of the proteoliposomal membrane, the substrate specificity of the microsomal ANTS is confined essentially to adenine nucleotides and, to a lesser extent, to guanine nucleotides.

The effects of inhibitors on the [14C]ATP/ATP exchange reaction catalysed by the reconstituted yeast ANTS were also examined (Fig. 5B). The lysine reagent, PLP, chosen as stop inhibitor (28, 47), caused the complete inhibition of ANTS function. As many other transport proteins, it was sensitive to organic mercurials, being virtually completely blocked by 0.1 mM HgCl₂ and 0.1 mM mersallyl and highly inhibited by 0.1 mM N-ethylmaleimide and 0.1 mM p-chloromercuribenzoate. DIDS, the well-known and powerful inhibitor of the Sac1p-dependent RE ATP transporter (17), markedly decreased the ATP/ATP exchange activity when it was used at 1 mM concentration. Bongkrekic acid and carboxyatractyloside (13, 22) are known to be specific inhibitors of the mitochondrial AAC. The use of 10 μM bongkrekic acid and of carboxyatractyloside concentrations higher than 0.1 mM resulted in only slight inhibition, indicating no contamination of this fraction with mitochondrial/peroxisomal fractions (15, 48).

**Kinetic characteristics of the reconstituted yeast ANTS**

The kinetic constants of the partially purified ANTS were determined by measuring the initial transport rate at various external [14C]ATP concentrations in the presence of a constant saturating internal concentration of 10 mM ATP. The \( K_m \) and \( V_{max} \) values calculated from 13 independent experiments at 25°C were 0.28 ± 0.02 mM and 3.98 ± 0.2 μmol/min per mg of protein, respectively. In order to measure the affinity of ANTS for some of the well transported nucleotides, we checked their ability to inhibit the [14C]ATP/ATP exchange reaction when added to a fixed concentration together with various external [14C]ATP concentrations. ADP, GTP and GDP were competitive inhibitors of the [14C]ATP uptake because they increased the apparent \( K_m \) without changing \( V_{max} \) (not shown); the measured \( K_i \) values were 0.07 ± 0.01 mM, 0.42 ± 0.05 mM and 0.35 ± 0.03 mM, respectively (means of three experiments for each).

**Discussion**

The existence of an ATP transport system has already been demonstrated in eukaryotes (23, 24, 49). The transport of ATP from the cytosol to the ER lumen is essential for the protein translocation across the ER membranes since the transfer of the bound protein precursors into the translocation pore and the subsequent transport is mediated by ATP-dependent ER chaperones (6, 50), as well as glycosylation or phosphorylation of many proteins, proteoglycans and lipids, occurring in the ER lumen, are all ATP-dependent reactions (7).

Although the presence of a Sac1p-dependent ATP/ADP exchange activity in the yeast microsomal membranes has already been reported (23, 24), its function
as a ER ATP/ADP transporter has been excluded since the reconstituted recombinant Sac1p did not catalyse any ATP/ADP exchange activity (24). On this basis, we have decided to shed light on this transport system. In order to unravel the role of Sac1p in the ER nucleotide transport, microsomal membranes from the wild-type and sac1Δ yeast strains were reconstituted into liposomes. Differently from what previously reported (23, 24), in our experimental conditions both membrane extracts from wild-type and sac1Δ ER catalysed the same ATP/ADP exchange activity and showed the same sensitivity to CAT and DIDS (Fig. 1B). These results clearly demonstrate that Sac1p has nothing to do with the measured ER ATP/ATP exchange activity. Similar results were previously found in the functional characterization of the ATP transporter from rat liver RE (20), where a 56 kDa purified protein was identified as responsible for an ATP/ADP exchange activity in the RE; no band at 67 kDa, weight typical of the mammalian SAC1 (51), was detected in the purified fraction (20). In order to identify the yeast ER ANTS and to reduce the huge amount of proteins present in the ER extract which could interfere with the reconstitution of the ATP transporter into liposomes, a purification attempt was carried out. By applying TX-100-solubilized sac1Δ ER extract on an HTP column, we recovered two active fractions (Fig. 3A, lanes 3 and 4), which essentially showed the same electrophoretic pattern and a similar ATP/ATP exchange activity (Fig. 3B). The two purified fractions were about 950-fold enriched with respect to the ATP/ATP exchange activity and contained four bands, one at 30 kDa, another at 56 kDa and a double band at 65–66 kDa. The 56 kDa band could represent the putative yeast orthologue of the rat microsomal ATP transporter, since a similar purification pattern was previously obtained during the identification of the rat liver microsomal ATP transporter, with a molecular weight of 56 kDa (20); in that work, using a photoaffinity labelling approach, the authors established that the 30 kDa band could be due to the degradation of the 56 kDa band. Furthermore, the high degree of homology very often found between yeast and mammalian orthologues (52) tipped the scales in favour of the 56 kDa band, since the same MW was found for the rat liver ER ATP transporter (20).

Several attempts, to further purify the yeast ER transporter by using different kind of chromatography failed, most probably due to the low levels of protein recovered in the HTP eluates. For this reason, the further biochemical characterization of the yeast ER ANTS was carried out on the HTP eluates. Before proceeding with these experiments, it was important to exclude that the ATP/ATP exchange activity measured in the HTP eluates was not due to the presence of a MCF member in the microsomal membranes, since among the four bands, visible on the SDS-PAGE of the HTP eluates, the one at 30 kDa was the most abundant and many members of the MCF have a MW ranging from 30 to 36 kDa (52). This doubt was soon ruled out, since the reconstituted microsomal membranes of the yeast strains carrying single deletions for each of the biochemically not characterized MCs showed the same ATP/ATP exchange activity as that of the sac1Δ ER extract (Fig. 3C). The tryptic digest from 56 kDa gel band was examined in MS reflection mode, whereas MS/MS experiments were used for peptide sequence discrimination. The human keratins originate from chemicals and/or sample handling become ubiquitous at low levels, as in the context of silver-stained gels. The pattern of keratin peptides is not visible on the SDS-PAGE of the HTP eluates, the one at 30 kDa, another at 56 kDa and a double band at 65–66 kDa. The 56 kDa band could represent the putative yeast orthologue of the rat microsomal ATP transporter, since a similar purification pattern was previously obtained during the identification of the rat liver microsomal ATP transporter, with a molecular weight of 56 kDa (20); in that work, using a photoaffinity labelling approach, the authors established that the 30 kDa band could be due to the degradation of the 56 kDa band. Furthermore, the high degree of homology very often found between yeast and mammalian orthologues (52) tipped the scales in favour of the 56 kDa band, since the same MW was found for the rat liver ER ATP transporter (20).

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Imd2p [Saccharomyces cerevisiae YJM1342] (Supplementary Figs S2 and S3), sequence coverage of 19%. MS/MS (53, 54) evaluation assigned a total of 2 peptides to the AJV32483.1, Imd2p (Saccharomyces cerevisiae YJM1342) (Supplementary Fig. S4). Further database searches did not retrieve any significant matches and we therefore concluded that band 56 kDa contained mainly one YJM1342. Imd2p is inosine-5’-monophosphate dehydrogenase, catalysing the conversion of inosine 5’-phosphate (IMP) to xanthosine 5’-phosphate (XMP). The identification of the band was unambiguous since the other retrieved proteins identified had significantly fewer matching tryptic peptides, a different intact molecular weight, and were from a different species. MS and MS/MS data suggest that the identification of the protein responsible for the transport activity was unsuccessful probably due to the very low amounts present in the sample.

The biochemical characterization of the partially purified ER ANTS was carried out by following a procedure successfully used to identify most of the mitochondrial transport systems (28, 55) including the mitochondrial (13, 22) and peroxisomal (14, 15) ADP/ATP transporters.

Interestingly, ANTS efficiently exchanged ATP not only when ATP or ADP were present inside the proteoliposomes, but also in the presence of internal AMP, dATP, dADP dAMP and guanine nucleotides (Fig. 5A). A guanine nucleotides transport by ANTS had never been described up to date. Furthermore, ANTS substrate specificity is not shared with any of the functionally known MCs, ruling out, once again, the most probable candidate of the yeast ER ANTS, further studies are required to confirm its identity.

Similarly to the yeast Sac1p-dependent ATP transporter, the partially purified ANTS showed a significant inhibitor sensitivity toward DIDS and was scarcely affected by carbamoylactylcide and bongkrekic acid, two potent inhibitors of the mitochondrial adenine nucleotide carrier (22, 57) (Fig. 5B).

Lastly, it should be noted that the affinity towards ATP showed by the partially purified ANTS was about 30-fold lower than that measured for the yeast Sac1p-dependent and the rat liver microsomal ATP transporter (20, 23). The Km for ATP measured in our experimental conditions was 0.28 mM whereas those previously found for the yeast and rat microsomal ATP transporter were about 10 μM. One possible explanation of this difference may reside in the different measuring conditions used to determine the kinetic constants. They are usually determined by measuring the initial transport rate at various external labelled substrate concentrations. These measurements should be taken at very short interval of time, when the transport rate is a linear function of time. Thus it is important to block all samples (having different external substrate concentrations) instantaneously, completely and at the same time in order to get reliable results, here we used the technique of the stop-inhibitor (28), which guarantees all these conditions; differently, the kinetic parameters previously measured for the yeast and rat liver ER transporter (20, 23) were determined by blocking the exchange reactions by the filtration technique which requires some seconds. It should also be emphasized that a Km for ATP in a millimolar range, found in this work, was also demonstrated for the ATP transporter of the chromaffin granules (56).

Furthermore, a Km for ATP in a millimolar range would be physiologically more relevant, since cytosolic ATP concentration is in the same range (58). A micromolar Km for ATP was found for the yeast mitochondrial adenine nucleotide carrier (48), but in this case it is physiologically relevant, since ATP concentration in the mitochondrial matrix is much lower than that of the cytosol (59), and it should be kept low in order to keep the oxidative phosphorylation going.

This report represents the first partial purification of the yeast ER ANTS completely independent on the presence of Sac1p. ANTS functional characterization into liposomes suggests that its main physiological role could be to transport ATP in the ER lumen in exchange for ADP, playing a crucial role in the great variety of ATP-requiring reactions occurring in the ER. Although, a 56 kDa band has been identified as the most probable candidate of the yeast ER ANTS, further studies are required to confirm its identity.

Supplementary Data

Supplementary Data are available at JB Online.

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Conflict of Interest

None declared.

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