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Molecular identification and functional characterization of a novel glutamate transporter in yeast and plant mitochondria

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ABSTRACT

The genome of *Saccharomyces cerevisiae* encodes 35 members of the mitochondrial carrier family (MCF) and 58 MCF members are coded by the genome of *Arabidopsis thaliana*, most of which have been functionally characterized. Here two members of this family, Ymc2p from *S. cerevisiae* and BOU from *Arabidopsis*, have been thoroughly characterized. These proteins were overproduced in bacteria and reconstituted into liposomes. Their transport properties and kinetic parameters demonstrate that Ymc2p and BOU transport glutamate, and to a much lesser extent L-homocysteinesulfinate, but not other amino acids and many other tested metabolites. Transport catalyzed by both carriers was saturable, inhibited by mercuric chloride and dependent on the proton gradient across the proteoliposomal membrane. The growth phenotype of *S. cerevisiae* cells lacking the genes *ymc2* and *agc1*, which encodes the only other *S. cerevisiae* carrier capable to transport glutamate besides aspartate, was fully complemented by expressing Ymc2p, Agc1p or BOU. Mitochondrial extracts derived from *ymc2Δagc1Δ* cells, reconstituted into liposomes, exhibited no glutamate transport at variance with wild-type, *ymc2Δ* and *agc1Δ* cells, showing that *S. cerevisiae* cells grown in the presence of acetate do not contain additional mitochondrial transporters for glutamate besides Ymc2p and Agc1p. Furthermore, mitochondria isolated from wild-type, *ymc2Δ* and *agc1Δ* strains, but not from the double mutant *ymc2Δagc1Δ* strain, swell in isosmotic ammonium glutamate showing that glutamate is transported by Ymc2p and Agc1p together with a H^+. It is proposed that the function of Ymc2p and BOU is to transport glutamate across the mitochondrial inner membrane and thereby play a role in intermediary metabolism, C1 metabolism and mitochondrial protein synthesis.

1. Introduction

Many metabolic pathways require a protein-mediated flux of solutes across cell membranes for their functioning. Ymc1p and Ymc2p from *Saccharomyces cerevisiae* and A bout de souffle (BOU) proteins belong to the mitochondrial carrier family (MCF) as they show all the features of this superfamily, namely a tripartite structure consisting of three related 100-residue domains, each containing the conserved signature motif PX[D/E]XX[K/R]X[K/R]X$_{20\text{-}30}$[D/E]GXXXX[W/Y/F][K/R]G (PROSITE PS50920, PFAM PF00153 and IPR00193) and two hydrophobic transmembrane segments connected by a long hydrophilic matrix loop [1–3]. Furthermore, these three transporters are localized to mitochondria [4,5]. Members of the MCF transport a large variety of solutes highly differing in size and nature, including amino acids, carboxylates, ketoacids, nucleotides, dinucleotides, inorganic ions and coenzymes [6,7].

The two homologous genes *ymc1* and *ymc2* are important for the utilization of oleic acid and the metabolism of glutamate in *Saccharomyces cerevisiae* [5]. Furthermore, given that Ymc1p and Ymc2p, which share 65% identical amino acid, suppress the oleic acid growth defect of strains lacking *odc1* and *odc2* [5], Trotter et al. (2005) suggested that Ymc1p and Ymc2p "possess at least a partial ability to transport similar substrates as Odc1p and Odc2p", the most efficiently transported of which are α-oxoglutarate and α-oxoadipate [8]. The

Abbreviations: MC, mitochondrial carrier; MCF, mitochondrial carrier family

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most closely related protein to Ymc1p and Ymc2p in *Arabidopsis thaliana* is BOU which was first suggested to function as a carnitine/acetylcarnitine carrier [4]. Later, this protein was found to be involved in photorespiration [9]. In fact, it is co-expressed with many photorespiratory genes among which GDP1, GDP2, GDT1 and GDH3, which encode subunits of the mitochondrial enzyme glycine decarboxylase (GDC1); deletion of the BOU gene causes a typical photorespiratory growth phenotype with an elevated CO_2 compensation point and accumulation of glycine; and in the BOU knockout mutant a degradation of a GDC1 subunit was shown together with a marked decrease in GDC1 activity. In view of the typical phenotype and the excessive glycine accumulation [9], Eisenhut et al. (2013) suggested that the BOU protein transports one of the metabolites necessary for proper GDC1 activity into mitochondria, i.e. malonate and pyruvate (required for lipoic acid synthesis), glutamate, para-aminobenzoate and pterin (required for tetrahydrofolate (THF) synthesis).

In the current study, we provide direct evidence that the gene products of YBR104w, named Ymc2p, and of At5g46800, named BOU, are mitochondrial transporters for L-glutamate in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, respectively. Ymc2p and BOU were overexpressed in *Escherichia coli*, and the gene products were purified, reconstituted in phospholipid vesicles, and shown to transport L-glutamate with high specificity by both counter-exchange and uniport mechanisms. *S. cerevisiae* cells lacking the genes *ymc2* and *agc1* exhibited a marked growth phenotype on acetate, which was fully restored by the individual expression of Ymc2p, Agc1p or BOU. Mitochondria isolated from wild-type, *ymc2*Δ and *agc1*Δ, but not from *ymc2*Δ*agc1*Δ, swell in isosmotic ammonium glutamate showing that glutamate is transported by Ymc2p and Agc1p together with a H^+. This is the first time that proteins capable of transporting almost exclusively L-glutamate have been identified at the molecular level in *S. cerevisiae* and Arabidopsis.

2. Materials and methods

2.1. Sequence search and analysis

Protein databases for *metazoa*, *fungi* and *plants* were screened with the protein sequences of Ymc1p, Ymc2p and BOU using BLASTP. Multiple sequence alignments were made with ClustalW and phylogenetic trees were constructed by the neighbor-joining method with MEGA7 [10].

2.2. Construction of expression plasmids

The coding sequences of *ymc1* (YPR058w), *ymc2* (YBR104w) and *BOU* (At5g46800) were amplified by PCR from *S. cerevisiae* genomic DNA (*ymc1* and *ymc2*) and an Arabidopsis cDNA library (At5g46800) [11]. Forward and reverse oligonucleotide primers were synthesized corresponding to the extremities of the coding sequences with additional *Bam*HI and *Hin*dIII (*ymc1* and *ymc2*) and *Eco*RI and *Xho*I (At5g46800) restriction sites. The amplified products were cloned into the pMW7 (*ymc1* and *ymc2*) or pRUN (At5g46800) expression vector and the constructs were transformed into *Escherichia coli DH5*α (*ymc1* and *ymc2*) or C0214(DE3) (At5g46800). The ymc2-pRS416 and BOU-pRS416 plasmids were constructed by cloning DNA fragments of about 1800 bp containing the open reading frame, about 720 bp upstream and 220 bp downstream of the ymc2 or BOU open reading frame, respectively (amplified from *S. cerevisiae* genomic DNA or an Arabidopsis cDNA library by PCR using primers with additional *Hin*dIII and *Bam*HI sites) into the low-copy centromeric vector pRS416 [12]. The BOU-pYES2 plasmid was constructed by cloning the coding sequences of BOU into the yeast pYES2 expression vector (Invitrogen) under the control of the constitutive *MIR1* promoter. The pRS416 and pYES2 vectors, prepared as above, were transformed into *E. coli* DH5α cells. Transformants were selected on 2xTY plates containing ampicillin (100 μg/ml) and screened by direct colony PCR and by restriction digestion of purified plasmids. The sequences of the insert were verified by DNA sequencing.

2.3. Bacterial expression and LC-MS/MS analysis of purified Ymc1p, Ymc2p and At5g46800 BOU

Ymc1p, Ymc2p and BOU were overexpressed at 37 °C as inclusion bodies in the cytosol of *E. coli* C0214(DE3) cells as previously described [13–16]. Control cultures with the empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient and washed first at 4 °C with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.0), then once with a buffer containing Triton X-114 (3%, w/v), 1 mM EDTA, 20 mM Na_2SO_4 and 10 mM PIPES–NaOH pH 7.0, and finally twice with TE buffer [17]. Afterwards proteins were separated on SDS-PAGE and stained with Coomassie blue. The gel slice containing the recombinant protein was washed sequentially for 15 min three times each step with: 1) acetonitrile (ACN) 50%, 2) 25 mM ammonium bicarbonate (NH_4HCO_3). Disulfide bridges were reduced using 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 56 °C. The reduced cysteines were alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 min in the dark and then washed twice for 15 min each with 100 mM NH_4HCO_3. Gel slices were dehydrated with ACN 100% for 5 min followed by vacuum centrifugation, incubated with the digestion buffer containing Sequencing grade Trypsin (Promega, Madison, WI, USA) in 20 mM ammonium bicarbonate for 1 h at room temperature and then overnight at 37 °C. The supernatant was transferred to a low protein binding tube and tryptic peptides were extracted from the gel slices using sequentially ACN 50% and trifluoroacetic acid (TFA) 5% for 30 min. The peptides were desalted using Stage Tips with C18 disks (Sigma Aldrich) resuspended in 0.1% formic acid (FA) before analysis using a LC-MS/MS system. The peptides were loaded on a C18 trap column and separated onto a C18 analytical column with a gradient from 100% mobile phase A (0.1% FA) to 35% phase B (0.1% FA, 95% ACN). The mass spectrometer was operated in positive ion mode with data-dependent acquisition. The 5 most intense ions were selected and fragmented with CID. The LC-MS/MS raw data were analyzed by database search algorithm embedded in Mascot (http://matrixscience.com). The tolerance on parents was 20 ppm and on fragments was 0.05 Da. The modifications allowed were oxidation on methionine as variable modification, and carbamidomethylation on cysteine as fixed modification. Database search of the acquired MS/MS spectra confirmed unambiguously the identity of the recombinant protein to which all selected ions belonged. The false discovery rate was below 0.1%, using a decoy database.

2.4. Reconstitution of Ymc1p, Ymc2p and BOU into liposomes and transport measurements

The inclusion body derived proteins were solubilized in 1.4% lauric acid (Ymc1p and Ymc2p) or 1.8% sarkosyl (BOU) (w/v), 10 mM PIPES (pH 7.0) and 3% Triton X-114. Unsolubilized material was removed by centrifugation (20,800 × *g* for 20 min at 4 °C). The solubilized recombinant proteins were reconstituted into liposomes by cyclic removal of the detergent with a hydrophobic column of amberlite beads (Bio-Rad), as previously described [18] with some modifications. The initial reconstitution mixture contained solubilized proteins (about 30 μg of Ymc1p and Ymc2p or 20 μg of BOU), 75 μl of 10% Triton X-114, 100 μl of 10% egg yolk phospholipids (Fluka) as sonicated liposomes [19], 10 mM substrate except where otherwise indicated, 0.7 mg (Ymc1p and Ymc2p) or 0.4 mg (BOU) cardiolipin, 20 mM PIPES (pH 7.0) and water to a final volume of 700 μl. These components were mixed thoroughly, and the mixture was recycled 13 times through an Amberlite (Bio-Rad) column (3.5 cm × 0.5 cm) pre-equilibrated with 10 mM PIPES (pH 7.0) and 50 mM NaCl (buffer A), and the substrate at the same concentration used in the starting mixture. External substrate was removed from

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proteoliposomes on Sephadex G-75 columns pre-equilibrated with buffer A. Transport at 25 °C was initiated by adding L-[^{14}C(U)]glutamate (Perkin Elmer) to substrate-loaded (exchange) or unloaded (uniport) proteoliposomes, and terminated by adding 0.5 (Ymc1p and Ymc2p) or 0.2 (BOU) mM $HgCl_2$ which, at these concentrations, inhibits the activities of these proteins completely and rapidly, most likely by blocking key cysteine residues. In controls, the inhibitor was added at the beginning together with the radioactive substrate according to the "inhibitor-stop" method [20]. Finally, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75 pre-equilibrated with buffer A, and the radioactivity in the proteoliposomes was measured. The experimental values were corrected by subtracting control values. The initial transport rates were calculated from the radioactivity taken up by proteoliposomes after 2 min in the initial linear range of substrate uptake. For efflux measurements, proteoliposomes containing 2 mM glutamate were loaded with 15 μM L-[^{14}C (U)] glutamate by carrier-mediated exchange equilibration [21,22]. After 50 min, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75. Efflux was started by adding unlabeled external substrate or buffer A alone, and terminated by adding the inhibitor indicated above.

2.5. Yeast strains, media and growth conditions

BY4742 (wild-type) and knockout *S. cerevisiae* strains were purchased by the EUROFAN resource centre EUROSCARF (Frankfurt, Germany). Deletion strains were verified by PCR. Transformants were selected on SC medium without histidine. The wild-type and knockout strains were grown in rich YP medium, containing 2% bactopeptone and 1% yeast extract. All media were supplemented with a fermentable or a nonfermentable carbon source (2% glucose, 2% galactose, 3% glycerol, 2% ethanol, 2% DL-lactate, 2% pyruvate or 3% acetate). The final pH was adjusted to 4.5 or, with acetate or pyruvate, to 6.5 [23]. Mitochondria were isolated by standard procedures from cells grown in YP medium containing lactate until the early exponential phase (optical density between 1.0 and 1.5) was reached and then shifted to acetate for 6 h before harvesting.

2.6. Other methods

The amount of purified Ymc1p, Ymc2p and BOU proteins was estimated by laser densitometry of stained samples using carbonic anhydrase as protein standard [24,25]. The share of protein incorporated into liposomes was measured as described [26] and was about 13% of protein added to the reconstitution mixture. The rate of mitochondrial swelling was monitored by the decrease in A_{546} as described previously [27]. Yeast mitochondria (250 μg of protein) were added to 1 ml of 120 mM ammonium salts of various anions, 20 mM Tris, 1 mM EDTA, 5 μM rotenone and 0.1 μM antimycin (pH 7.4).

3. Results

3.1. Bacterial expression of Ymc2p and BOU

Ymc2p and BOU were expressed at high levels in *E. coli* C0214(DE3) (Fig. 1). They accumulated as inclusion bodies and were purified by centrifugation and washing (see Materials and Methods). The apparent molecular masses of the purified proteins (Fig. 1, lanes 3 and 6; yield 25–65 mg/l) were approximately 37.1 kDa for Ymc2p and 30.7 kDa for BOU (calculated values are 36,555 and 31,022 Da, respectively). Their identities were confirmed by LC-MS/MS. The proteins were not detected in cultures harvested immediately before the induction of expression (Fig. 1, lanes 1 and 4).

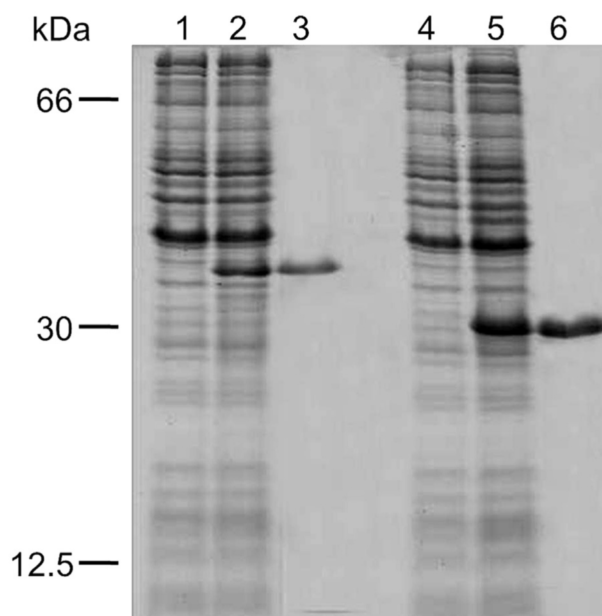

Fig. 1. Expression in *E. coli* and purification of Ymc2p and BOU. Proteins were separated by SDS-PAGE and stained with Coomassie Blue dye. The positions of molecular weight markers (bovine serum albumin, carbonic anhydrase and cytochrome *c*) are shown on the left; lanes 1–2 and 4–5, *E. coli* CO214(DE3) containing the expression vector with the coding sequence for Ymc2p (lanes 1 and 2) and BOU (lanes 4 and 5). Samples were taken at the time of the induction (lanes 1 and 4) and 5 h later (lanes 2 and 5). Equivalent samples were analyzed. Lanes 3 and 6, 2 μg of Ymc2p and BOU originated from bacteria shown in lanes 2 and 5, respectively.

3.2. Functional characterization of Ymc2p and BOU

Ymc1p, Ymc2p and BOU were reconstituted into liposomes, and their transport activities for substrates known to be transported by mitochondria were tested in homo-exchange (same substrate inside and outside) experiments. Using external and internal substrate concentrations of 1 and 10 mM, respectively, Ymc2p and BOU reconstituted proteins catalyzed an active [^{14}C]glutamate/glutamate exchange, which was inhibited completely by 0.2 and 0.5 mM mercuric chloride for BOU and Ymc2p, respectively. By contrast, despite the long time of incubation (i.e. 30 min) they did not catalyze homo-exchanges of aspartate, lysine, arginine, malate, oxoglutarate, ketoisocaproate, citrate, carnitine, ADP, glutathione, choline, spermine, proline, cysteine and threonine. Of importance, no [^{14}C]glutamate/glutamate exchange activity in liposomes reconstituted with Ymc2p and BOU was observed when Ymc2p and BOU were inactivated by boiling before incorporation into liposomes or when liposomes were reconstituted with lauric acid- or sarkosyl-solubilized protein from bacterial cells either lacking the expression vector or harvested immediately before induction of expression (data not shown). Likewise, no such activity was detected in liposomes reconstituted with two unrelated MCs, Ggc1p [28] and Oac1p [29,30], which had been expressed and purified from *E. coli*. Furthermore, the [^{14}C]glutamate/glutamate homo-exchange was null using pure liposomes, i.e. without incorporated protein (data not shown). At variance with Ymc2p and BOU, recombinant and reconstituted Ymc1p showed no activity under any of the experimental conditions tested, which include variation of the parameters that influence solubilization of the inclusion bodies and reconstitution of the protein into liposomes.

The substrate specificity of recombinant Ymc2p and BOU was further examined in-depth by measuring the uptake of [^{14}C]glutamate into Ymc2p- and BOU-reconstituted liposomes that had been preloaded internally with a high concentration (10 mM) of various potential

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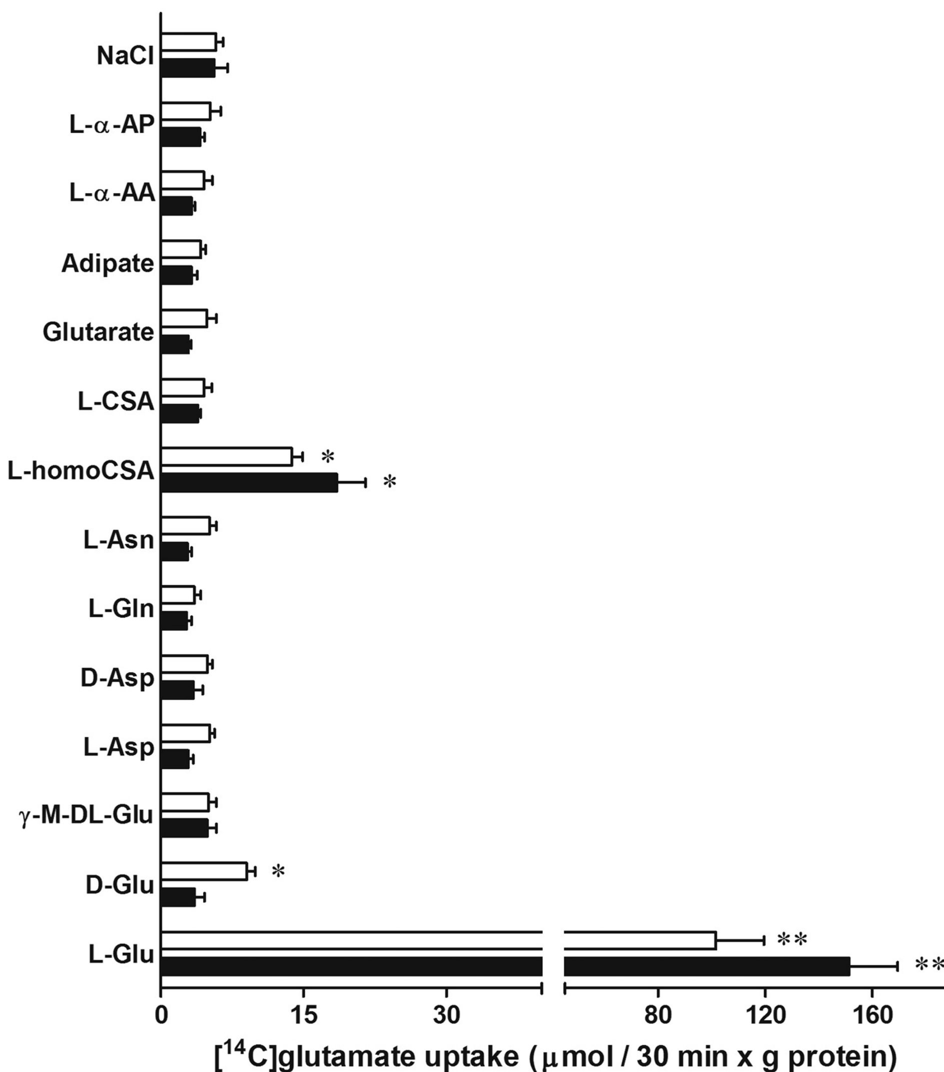

Fig. 2. Substrate specificity of Ymc2p and BOU. Liposomes were reconstituted with Ymc2p (black bars) or BOU (white bars) and preloaded internally with various substrates (concentration, 10 mM). Transport was started by adding 0.25 mM [^{14}C]glutamate and terminated after 30 min. The values are means ± SEM of at least three independent experiments. Differences between the activities of glutamate uptake with internal L-glutamate, L-homo-cysteinesulfinate and D-glutamate (with BOU) and the activity with internal NaCl and no substrate are significant (**$p < 0.01$ and *$p < 0.05$, Student's *t*-test). Differences between the activity of glutamate uptake with internal NaCl and the activity with internal substrates other than L-glutamate, L-homo-cysteinesulfinate, and D-glutamate (with BOU) are not significant ($p > 0.05$, Student's *t*-test). Abbreviations: γ-M-DL-Glu, γ-methyl-DL-glutamate; L-CSA, L-cysteinesulfinate; L-homoCSA, L-homo-cysteinesulfinate; L-α-AA, L-α-aminoadipate; L-α-AP, L-α-aminopimelate.

substrates (Fig. 2). With both proteins, external L-glutamate exchanged very efficiently only in the presence of internal L-glutamate. A significant but low exchange, about one order of magnitude lower than the glutamate/glutamate exchange, was observed in the presence of internal L-homocysteinesulfinate and even lower with internal D-glutamate (with BOU). Virtually no exchange was found with the structurally related compounds D-glutamate (with Ymc2p), α-methylglutamate, L-aspartate, D-aspartate, L-glutamine, L-asparagine, L-cysteinesulfinate, glutarate, adipate, L-α-aminoadipate, L-α-aminopimelate (Fig. 2) and with ornithine, lysine, arginine, histidine, cysteine, serine, threonine, glycine, valine, proline, carnitine, acetylcarnitine, fumarate, malonate, succinate, malate, pimelate, citrate, phosphate, sulfate, oxoglutarate, ATP, oxaloacetate, pyruvate, phosphoenolpyruvate, citrulline, taurine and tartrate (data not shown). The residual activity in the presence of these substrates was approximately the same as the activity observed in the presence of NaCl and no substrate. Therefore, among the many compounds tested, reconstituted Ymc2p and BOU transport only L-glutamate and, to a lesser extent, L-homocysteinesulfinate.

3.3. Kinetic characteristics of recombinant Ymc2p and BOU

In Fig. 3, the time courses of 1 mM [^{14}C]glutamate uptake into liposomes reconstituted with Ymc2p (A) and BOU (B), measured either as exchange (in the presence of 10 mM glutamate) or as uniport (with internal NaCl), are compared. All data sets followed first-order kinetics with isotopic equilibrium being approached exponentially. The ratio of maximal substrate uptake by the exchange and by the uniport was approximately 10 for both Ymc2p and BOU, in agreement with the expected value from the intraliposomal concentrations at equilibrium (10 and 1 mM for exchange and uniport, respectively). The initial rates of glutamate uptake were 14.8 and 2.4 μmol/min × *g* of protein (Ymc2p) and 9.2 and 2.8 μmol/min × *g* of protein (BOU) for the exchange and the uniport, respectively. The uniport mode of transport was further investigated by measuring the efflux of [^{14}C]glutamate from prelabeled active proteoliposomes because it provides a more convenient assay for unidirectional transport [18]. As shown in Fig. 4, a substantial efflux of [^{14}C]glutamate from liposomes reconstituted with Ymc2p (A) or BOU (B) was observed with the addition of buffer A alone, i.e. in the absence of external substrate. Furthermore, a greater efflux of [^{14}C]glutamate occurred upon the addition of unlabeled glutamate. Finally, both effluxes, i.e. with and without external substrate, were prevented completely if the inhibitor mercuric chloride was present from the beginning of the proteoliposome incubation (time 0). These results show that recombinant Ymc2p and BOU are capable of catalyzing both the uniport of glutamate and the glutamate/glutamate exchange.

The kinetic constants of recombinant Ymc2p and BOU were determined from the initial transport rates of the [^{14}C]glutamate uptake at various external labeled substrate concentrations in the presence of 10 mM internal glutamate (exchange) or 10 mM NaCl. The Michaelis constants (Km) of Ymc2p and BOU for glutamate were 15.2 ± 1.8 μM

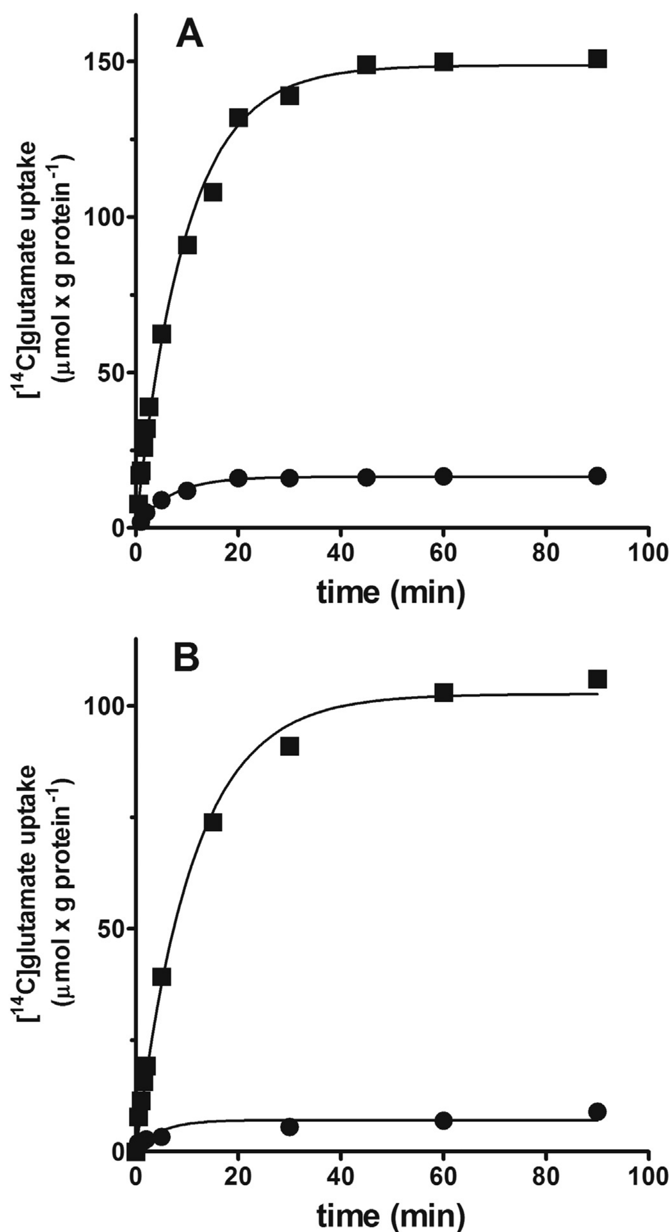

Fig. 3. Time courses of [14C]glutamate/glutamate exchange and [^{14}C]glutamate uniport by Ymc2p and BOU. Proteoliposomes were reconstituted with Ymc2p (panel A) or BOU (panel B) and contained 10 mM glutamate (exchange, ■) or 10 mM NaCl and no substrate (uniport, ●). Transport was initiated by adding 1 mM [^{14}C]glutamate to proteoliposomes. Similar results were obtained in three independent experiments.

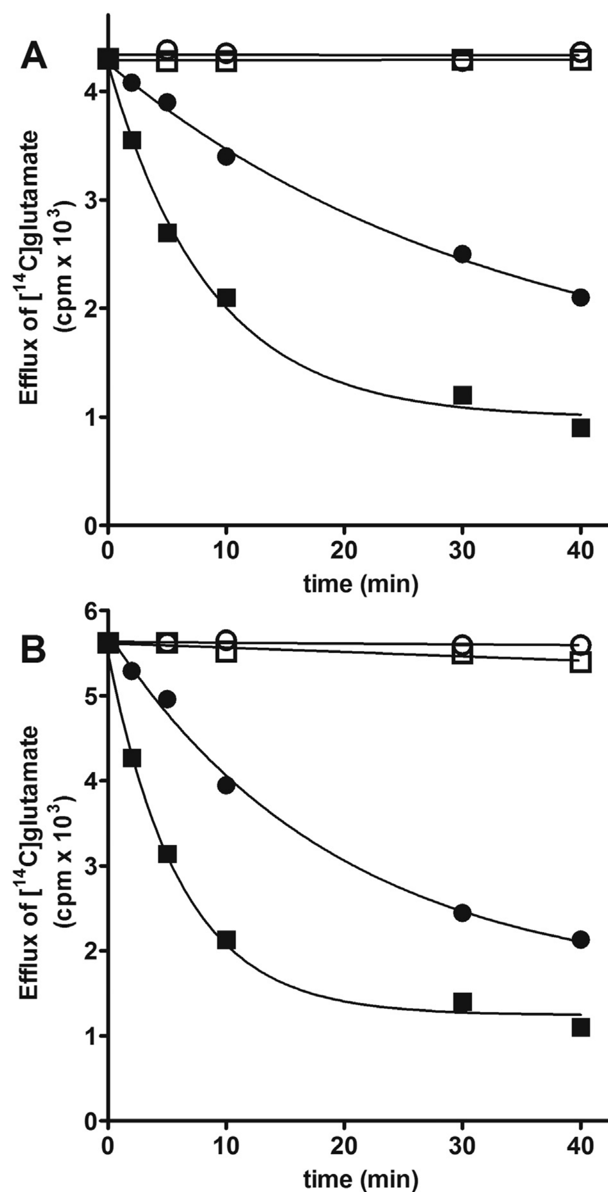

Fig. 4. Efflux of [^{14}C]glutamate from proteoliposomes. Liposomes were reconstituted with Ymc2p (A) or BOU (B) and preloaded internally with 2 mM glutamate. The internal substrate pool was labeled by carrier-mediated exchange equilibration. Then, the proteoliposomes were passed through Sephadex G-75. The efflux of [^{14}C]glutamate was started by adding buffer A alone (filled circles), 0.2 mM (BOU) or 0.5 mM (Ymc2p) mercuric chloride in buffer A (open circles), 5 mM glutamate in buffer A (filled squares) or 5 mM glutamate and 0.2 mM (BOU) or 0.5 mM (Ymc2p) mercuric chloride in buffer A (open squares). Similar results were obtained in three independent experiments.

and 25.3 ± 3.2 μM, respectively. The maximal activities (Vmax) of the glutamate/glutamate homo-exchange were 16.3 ± 2.7 and 9.3 ± 1.8 μmol/min × *g* of protein for Ymc2p and BOU, respectively, whereas the corresponding Vmax of glutamate uniport were 3.5 ± 1.0 and 3.8 ± 0.8 μmol/min × *g* protein.

3.4. Influence of pH gradient on the import of glutamate into liposomes reconstituted with Ymc2p or BOU

In the next step, the influence of the proton gradient on the uptake of glutamate into unloaded Ymc2p- and BOU-reconstituted liposomes was studied. In these experiments, the proton gradient across the proteoliposomal membrane was imposed as follows. The proteoliposomes were reconstituted at various pH values (from 6.5 to 8.0). After removal of the external buffer by passage through Sephadex G-75, [^{14}C]glutamate (buffered at pH 6.5) was added to the proteoliposomes, and the initial rates of uptake were measured. The results, shown in Figs. 5 A and B, demonstrate that with both Ymc2p and BOU the rate of glutamate uptake increased several times on raising the internal pH from 6.5 to 8.0 (at a fixed external pH of 6.5). Notably, this increase in glutamate uptake on raising the internal pH was virtually abolished in the presence of FCCP. As a further control, we examined the dependence of the glutamate/glutamate exchange on the proton gradient imposed across the liposomal membrane using the same experimental conditions. Figs. 5 A and B demonstrate that with both Ymc2p and BOU the rate of glutamate/glutamate exchange was very little affected by the increase in the internal pH from 6.5 to 8.0 at a fixed external pH of 6.5. These

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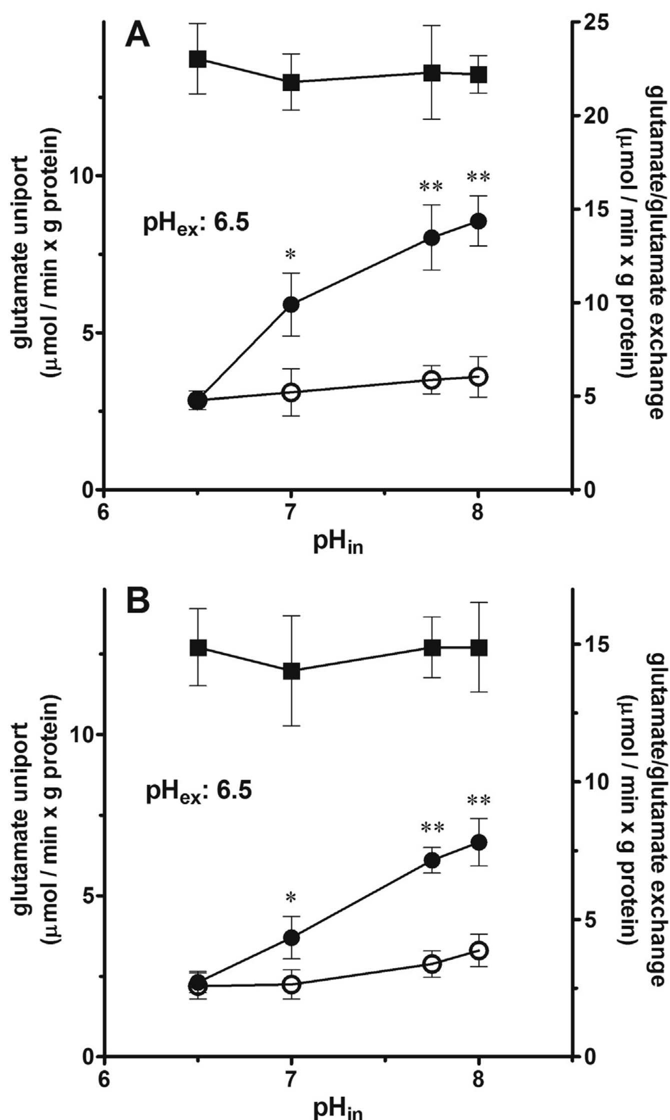

Fig. 5. Dependence on trans-membrane pH gradient of glutamate uniport by Ymc2p and BOU. The reconstitution mixture contained 20 mM glutamate (glutamate/glutamate exchange) or 10 mM NaCl and no substrate (glutamate/H^+ symport) and 50 mM MES/50 mM HEPES at the indicated pH values. After reconstitution of Ymc2p and BOU into liposomes, a mixture of 100 mM sucrose and 1 mM MES/1 mM HEPES at the same pH of the reconstitution mixture was used to equilibrate and to elute the Sephadex G-75 columns. Transport was started by adding 0.25 mM [^{14}C]glutamate together with 10 mM MES/10 mM HEPES at pH 6.5, in the presence and absence of 2 μM FCCP, to proteoliposomes reconstituted with Ymc2p (panel A) or BOU (panel B). The reaction was terminated after 2 min. [^{14}C]glutamate/glutamate exchange (■); [^{14}C]glutamate/H^+ symport (●); [^{14}C]glutamate/H^+ symport in the presence of FCCP (○). The values are means ± SEM of four independent experiments in duplicate for each carrier. In A and B differences between the activities of glutamate uniport at internal pH 7, 7.8 and 8.0 and the activities at internal pH 6.5 are significant (**$p < 0.01$ and *$p < 0.05$, Student's *t*-test); the same differences in the presence of FCCP as well as the differences between the activities of glutamate/glutamate exchange at internal pH 7, 7.8 and 8.0 and the activities at internal pH 6.5 are not significant ($p > 0.05$).

experiments, showing that alkalinization of the compartment opposite to where glutamate is present stimulates markedly the net transport of glutamate but influences scarcely the glutamate/glutamate exchange mediated by Ymc2p and BOU, clearly indicate that glutamate is transported across the reconstituted liposomal membrane together with H^+ by these proteins.

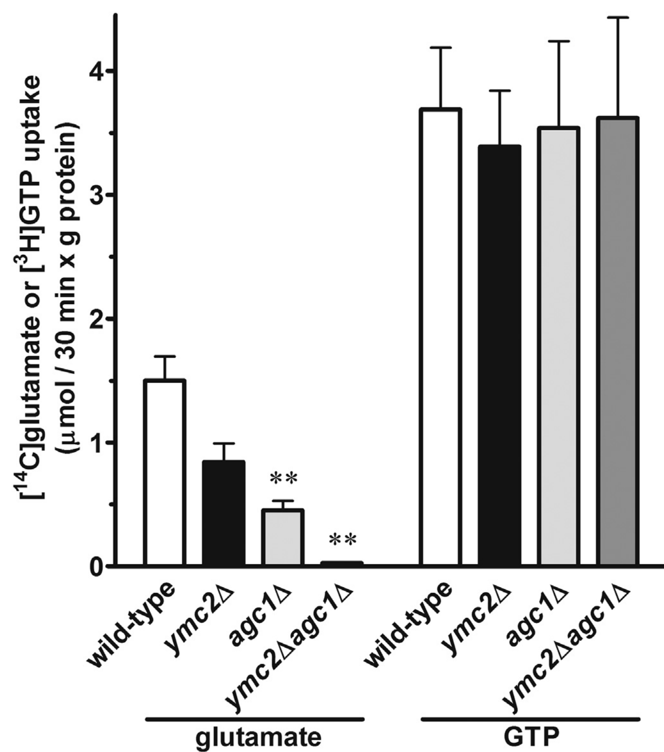

Fig. 6. Glutamate transport assays in liposomes reconstituted with mitochondrial extracts from wild-type and knockout *S. cerevisiae* strains. Whole mitochondria (0.8 mg of proteins × ml^{-1}) from the parental and *ymc2Δ*, *agc1Δ*, *ymc2Δagc1Δ* strains were solubilized in 2% Triton X-100, 50 mM NaCl, 1 mM EDTA and 10 mM PIPES (pH 7.0) for 20 min at 0 °C and centrifuged (138,000*g* for 10 min). Supernatants (about 5 μg of proteins) were reconstituted into liposomes and the indicated exchange were tested (internal substrate, 10 mM glutamate or GTP; external substrate, 100 μM L-[^{14}C]glutamate or 10 μM [^{3}H]GTP). The data represent means ± SEM of at least three experiments. Differences between [^{14}C]glutamate uptake with mitochondrial extracts from *agc1Δ* and *ymc2Δagc1Δ* strains and that with mitochondrial extracts from the parental strain are significant (**$p < 0.01$, Student's *t*-test). Differences between [^{3}H]GTP uptake with mitochondrial extracts from *ymc2Δ*, *agc1Δ* and *ymc2Δagc1Δ* strains and that with mitochondrial extracts from the parental strain are not significant ($p > 0.05$).

3.5. Effect of deleting ymc2 and/or other genes on the growth of S. cerevisiae cells

Having established the transport function of Ymc2p by *in vitro* assays, we decided to investigate the effect of deleting the genes *ymc1* and *ymc2* in yeast cells. In these experiments the *agc1Δ* and *ymc2Δagc1Δ* mutant strains were also employed because *S. cerevisiae* mitochondria contain another transporter for glutamate, the aspartate glutamate carrier (Agc1p), that catalyzes a counter-substrate exchange mode of transport, as the human AGC1-2 [31], and also a slower unidirectional transport (uniport) of substrates [32]. The deletion of the above mentioned genes was verified by diagnostic PCR performed by Euroscarf (SRD, scientific research and development). Furthermore, we measured the glutamate/[^{14}C]glutamate exchange in proteoliposomes that were reconstituted with Triton X-100 extracts of whole mitochondria from wild-type, *ymc2Δ*, *agc1Δ* and *ymc2Δagc1Δ* strains. A substantial reduction of the exchange was observed upon reconstitution of mitochondrial extracts from *ymc2Δ* or *agc1Δ* deleted strains, and no exchange at all was detected in proteoliposomes reconstituted with the mitochondrial extract from the double mutant *ymc2Δagc1Δ* strain as compared to the activity found using parental mitochondrial extract (Fig. 6). As a control, the GTP/[^{3}H]GTP exchange activity mediated by Ggc1p [28] was virtually the same in proteoliposomes reconstituted with the wild-type

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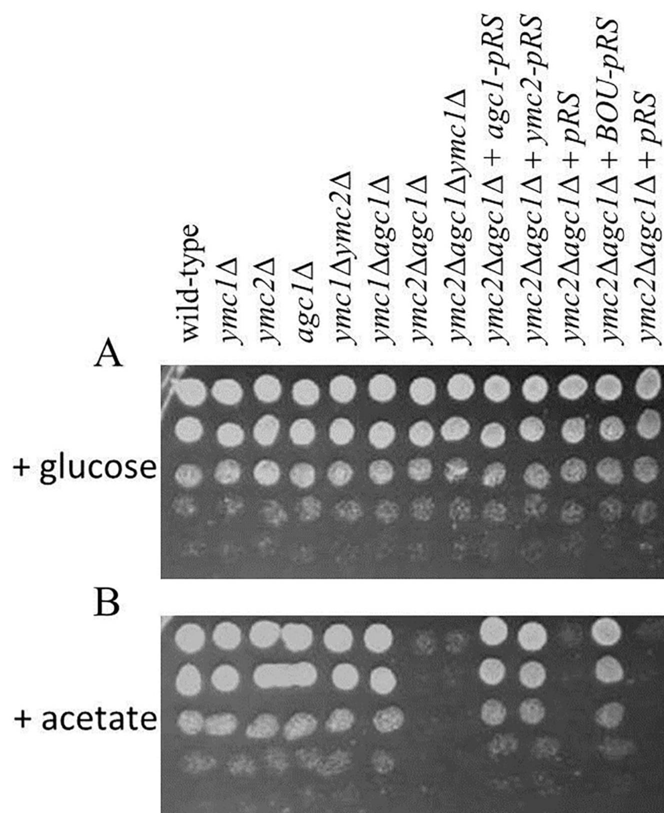

Fig. 7. Growth behavior of wild-type and various single and multiple deleted yeast strains. Fourfold serial dilutions of wild-type, *ymc1*Δ, *ymc2*Δ, *agc1*Δ, *ymc1*Δ*ymc2*Δ, *ymc1*Δ*agc1*Δ, *ymc2*Δ*agc1*Δ*ymc1*Δ cells and *ymc2*Δ*agc1*Δ cells transformed with the *agc1*-pRS416, *ymc2*-pRS416 or BOU-pRS416 plasmids were spotted on solid YP medium supplemented with 2% glucose or 2% acetate. Each row represents the sample and four 1:10 dilutions.

or deleted strains (Fig. 6). Then, the wild-type as well as the single mutants *ymc2*Δ, *ymc1*Δ and *agc1*Δ, the double mutants *ymc1*Δ*ymc2*Δ, *ymc1*Δ*agc1*Δ and *ymc2*Δ*agc1*Δ and the triple mutant *ymc2*Δ*agc1*Δ*ymc1*Δ strains were tested for growth on YP medium supplemented with either glucose or acetate. Interestingly, a marked retardation of growth was observed only in the double mutant *ymc2*Δ*agc1*Δ and the triple mutant *ymc2*Δ*agc1*Δ*ymc1*Δ on YP medium containing acetate, but not glucose (Fig. 7), lactate, pyruvate or ethanol (results not shown). Furthermore, the growth phenotype of the double mutant *ymc2*Δ*agc1*Δ was restored by complementing the deleted stain with the *ymc2*-pRS416 or *agc1*-pRS416, demonstrating that the phenotype of the double mutant on acetate-containing YP medium is the result of the absence of the two carriers Ymc2 and Agc1p and not a secondary effect. Notably, the expression of the BOU gene in the double knockout *ymc2*Δ*agc1*Δ under the same experimental conditions also rescued the growth defect (Fig. 7). The same growth restoration of the *ymc2*Δ*agc1*Δ mutant strain was obtained by using *ymc2*-, *agc1*- or BOU-pYES plasmids instead of pRS416 vectors.

3.6. Swelling of S. cerevisiae mitochondria from knockout strains

The transport of glutamate across the mitochondrial inner membrane can be studied by monitoring the swelling of mitochondria in the presence of an isoosmotic solution of ammonium glutamate. According to this method, introduced by [33] and employed by others ([34]; Crompton et al.,1974a; Crompton et al., 1974b; [35–40]), mitochondria swell in ammonium salts of anionic substrates only when the anion enters into the mitochondrial matrix together with protons. As illustrated in Fig. 8, wild-type, *ymc2*Δ and *agc1*Δ yeast mitochondria swelled in ammonium glutamate. The slight reduction in glutamate-induced swelling occurring with *ymc2*Δ and *agc1*Δ single mutant mitochondria as compared to that observed with wild-type mitochondria may indicate that both Agc1p and Ymc2p are required for maximal swelling. In contrast and noteworthily, mitochondria from the double deleted *ymc2*Δ*agc1*Δ strain did not swell in ammonium glutamate, indicating that there are no further H^+-compensated mitochondrial glutamate transporters in *S. cerevisiae* cells grown in the presence of acetate besides Agc1p and Ymc2p. Furthermore, all types of mitochondria did not swell in NH_4^+-chloride, as chloride is not transported together with H^+, whereas they all showed rapid swelling in isoosmotic solution of ammonium phosphate, indicating the presence of a very active H^+-phosphate symporter and the integrity of the mitochondria. Given that NH_4^+ penetrates the mitochondrial membrane as NH_3, these results show that glutamate$^-$ is transported into the mitochondria together with a H^+ forming NH_4^+ inside the organelles, just as it occurs in isosmotic solutions of ammonium phosphate or acetate.

3.7. Bioinformatics analysis

A previous phylogenetic tree of all MCs in *Homo sapiens*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* [7] revealed that Ymc1p, Ymc2p and BOU are located in a clade containing the ornithine carriers (human ORC1-2 and yeast Orc1p) [40,41], the plant carriers for basic amino acids (BAC1 and BAC2) [24,42], the carnitine/acylcarnitine carriers (human CAC and yeast Crc1p [43–45] and the arginine lysine carrier (human SLC25A29) [26] and the unidentified human SLC25A45 and SLC25A47. The present targeted phylogenetic analysis of ORC1-2, yeast Orc1p, BAC1-2, Ymc1p, Ymc2p, BOU, SLC25A29, the carnitine/acylcarnitine carriers (human CAC and yeast Crc1p), the glutamate carriers (human GC1-2 and *Drosophila melanogaster* GC1-2) [46,47] and the aspartate-glutamate carriers (human AGC1-2 and yeast AGC1) [31,32], consistently identified two well-separated sequence clades for basic and acidic amino acids (Fig. 9). Furthermore, Ymc1p, Ymc2p and BOU were phylogenetically closer to each other than the carriers for basic amino acids and the carnitine carriers. Therefore, Ymc1p, Ymc2p and BOU differ phylogenetically not only from GC1-2, Agc1p and AGC1-2, but also from the transporters for basic amino acids and the carnitine carriers, although they share with the latter a common ancestor sequence.

4. Discussion

In this work the transport properties of Ymc1p, Ymc2p and BOU have been investigated by recombinant expression, purification and reconstitution into liposomes (the EPRA method [6]) as well as by the phenotype analysis of yeast knockout cells. The transport properties and kinetic characteristics of recombinant and reconstituted Ymc2p and BOU, together with their mitochondrial localization [4,5], demonstrate that these proteins are mitochondrial transporters for glutamate in *S. cerevisiae* and *Arabidopsis thaliana*, respectively. This is the first time that a MC specific for glutamate has been identified in these organisms. For the closely related sequence of Ymc1, which was also cloned and expressed in *E. coli* in this study, no biochemical data are available, as we were incapable of renaturing and/or reconstituting it functionally. Recently, evidence has been provided that Ymc1p is a potential secondary mitochondrial glycine transporter [48]. However, because of the high degree of sequence similarity between Ymc1p and Ymc2p (65% identical amino acids), which is similar to that found for other MC isoforms [40,46,49], together with the notion that both YMC1 and YMC2 complement the growth defect on oleate of a *S. cerevisiae* strain devoid of the two oxodicarboxylate transporter isoforms [5], it cannot be excluded at present that Ymc1 is an isoform of the glutamate transporter in *S. cerevisiae*.

Ymc2p and BOU share a number of similar transport properties. They both catalyze unidirectional transport of glutamate and, as

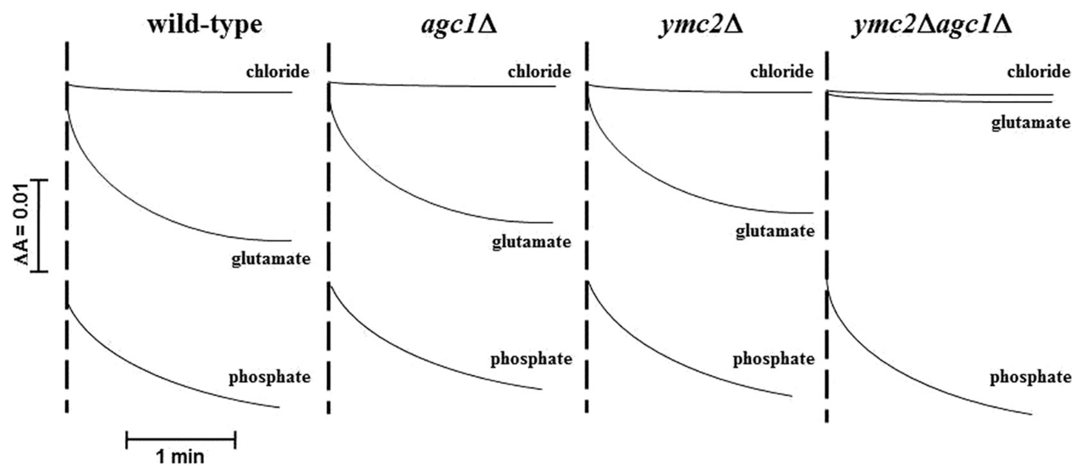

Fig. 8. Swelling of wild-type, *ymc2*Δ, *agc1*Δ and *ymc2*Δ*agc1*Δ yeast mitochondria in isoosmotic ammonium solutions of different anions. The incubation mixture contained 120 mM ammonium salts of the indicated anions, 20 mM Tris, 1 mM EDTA, 5 μM rotenone, 0.1 μM antimycin and mitochondria (0.25 mg of protein) in a final volume of 1 ml (pH 7.4). The turbidity changes of the mitochondrial suspensions were recorded at 546 nm. Temperature: 25 °C.

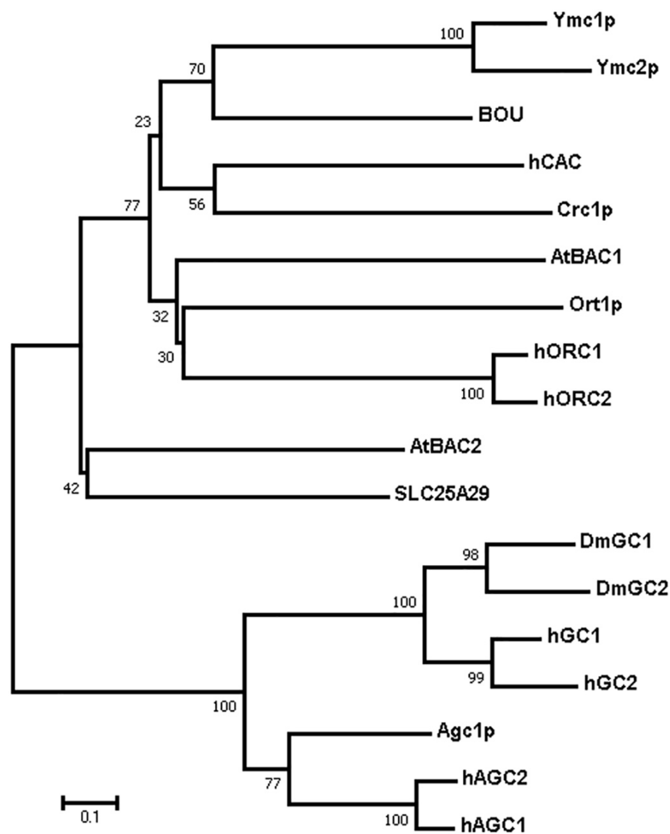

Fig. 9. The tree is originated from ClustalW multiple-sequence alignments by using the neighbor-joining method implemented in MEGA7. Bootstrap values for 1000 replicates are reported on each node. The bar indicates the number of substitutions per residue, with 0.1 corresponding to a distance of 10 substitutions per 100 residues. The proteins have the following accession numbers: *A. thaliana* AtBAC1, NP_180938; *A. thaliana* AtBAC2, NP_178108; *A. thaliana* BOU, NP_568670; *H. sapiens* hAGC1, NP_003696; *H. sapiens* hAGC2, NP_001153682; *H. sapiens* hCAC, NP_000378; *H. sapiens* hGC1, NP_001177990; *H. sapiens* hGC2, NP_001290413; *H. sapiens* hORC1, NP_055067; *H. sapiens* hORC2, NP_114153; *H. sapiens* SLC25A29, NP_001034444; *S. cerevisiae* Crc1p, NP_014743; *S. cerevisiae* Ortp1, NP_014773; *S. cerevisiae* Agc1p, NP_015346; *S. cerevisiae* Ymc1p, NP_015383; *S. cerevisiae* Ymc2p, NP_009662; *D. melanogaster* DmGC1 NP_650134; *D. melanogaster* DmGC2, NP_731657.

reported for all the other known MCs, a faster counter exchange mode of transport; have transport affinities (Km) lower than those exhibited by most characterized MCs; have specific activities (Vmax) similar to those found for the other MCs; and catalyze a transmembrane glutamate$^-$ + H$^+$ symport. However, the Vmax of the glutamate/glutamate exchange catalyzed by Ymc2p is almost double than the corresponding value of BOU although the Vmax value of the glutamate uniport is not significantly different.

Among the MCF members, the substrate specificity of Ymc2p and BOU differs from that of any other MC of known sequence and function, except for the glutamate carriers from humans [46] and *Drosophila melanogaster* [47]. In particular, they differ from other MCs that transport glutamate: the AGCs that also transport efficiently aspartate and cysteinsulfinate [31] and the plant UCP1-2 that also transport efficiently aspartate and several dicarboxylates [50]. Ymc2p and BOU also differ from the mitochondrial basic amino acid transporters and the carnitine carriers as they do not transport basic amino acids nor carnitine and acylcarnitines, although Ymc2p and BOU are phylogenetically related to these proteins [7,51]. Finally, they are definitely distinct from the high eukaryote glutamate carriers GC1-2, because Ymc2p and BOU are phylogenetically very distant as documented by phylogenetic analyses (Fig. 9 and [7]). Furthermore the % of identical amino acids between Ymc1-2 and BOU and GC1-2 (from both humans and *Drosophila melanogaster*) is only 22–27%, a value that is not greater than the basic homology existing between the different members of the MCF. Therefore, it may be assumed that their common function of transporting glutamate has been acquired through independent evolutionary routes.

It is noteworthy that the percentage of identical amino acids shared by Ymc2p and BOU (37%) is significant. Indeed, the percentage of identity between the yeast and the rat citrate carrier proteins is only 34% [52,53]. Apart from the homology that Ymc2p and BOU share with the transporters for basic amino acids and carnitine carriers ranging from 26 to 36% of identical amino acids, Ymc2p and BOU do not exhibit significant sequence homology with any other MC. However, several proteins encoded by the genomes of fungi are very likely orthologs of Ymc1p and Ymc2p. These sequences include: Q6CVY2 from *Klyveromyces lactis* (75% and 60% of identical amino acids with Ymc1p and Ymc2p, respectively); Q10248 from *Schizosaccharomyces pombe* (49% with both); Q6MFE5 and Q7S597 from *Neurospora crassa* (44% with both). Similarly, likely orthologs of BOU in monocots, dicots, conifers and mosses are: I1J4W1_SOYBN from *Glycine max* (80% of identical amino acids); B9N2N2_POPTR from *Populus trichocarpa* (79%); B8BID6_ORYSI from *Oryza sativa* (78%); A0A1S4DAN8_TOBAC *Nicotiana tabacum* (77%); I3T4H4_LOTJA *Lotus japonicus* (77%);

B4FEA2_MAIZE *Zea mays* (76%); B8LPX0_PICSI *Picea sitchensis* (74%); and A0A2K1IRF6_PHYPA *Physcomitrella patens* (70%). To our knowledge none of the above reported proteins has been characterized biochemically. On the contrary, it is unlikely that there are orthologous carriers of Ymc1p, Ymc2p and BOU in higher eukaryotes.

Given that the almost exclusive substrate for both Ymc2p and BOU is L-glutamate, a physiological function of these proteins is to catalyze the import uptake of glutamate into the mitochondria, where this metabolite is required for major processes occurring in the organelles. Notably, the entry of glutamate into energized mitochondria is favored because glutamate is co-transported with a proton by both Ymc2p and BOU and, therefore, is influenced by the chemical component (ΔpH) of the electrochemical force generated by the respiratory chain. In the mitochondrial matrix, due to the presence of the glutamate dehydrogenases Gdh2p and Gdh3p in *S. cerevisiae* [54] and of GDH1-3 in *Arabidopsis thaliana* [55,56], glutamate may be oxidized especially when cells meet specific metabolic states and growth conditions. Ymc2p and BOU may also play an important role in C1 metabolism [57–59]. In fact, in Arabidopsis glutamate can be utilized for the synthesis of tetrahydrofolate (THF) by the action of the mitochondrial dihydrofolate reductase (DHFR) and for its polyglutamylation. In *S. cerevisiae*, where THF is produced in the cytosol a primary function of Ymc2p is to provide the substrate within the mitochondria for the polyglutamylation of 5-methyl-THF and 10-formyl-THF catalyzed by folylpolyglutamate synthetase (Met7p). These reactions are fundamental for C1 transfer reactions because i) THF is a cofactor of the two Arabidopsis mitochondrial enzymes glycine decarboxylase (GDC) and serine hydroxymethyltransferase that catalyze the conversion of two glycine molecules into serine and are essential for photorespiration [60], and the *S. cerevisiae* mitochondrial enzyme serine hydroxymethyltransferase that converts one molecule of glicyne into serine; and ii) polyglutamylation of THF retains the folate within mitochondria, as well as within cells and other subcellular compartments, and plays a crucial role in maintenance homeostasis [61,62]. Another physiological function of Ymc2p and BOU is to provide the common substrate glutamate to the aspartate aminotransferase in both *S. cerevisiae* (AspAT) [63] and *Arabidopsis thaliana* [64] and to glutamine synthetase in *Arabidopsis* [60]. Furthermore, because mitochondria are equipped in their matrix with the entire machinery required for the synthesis of 8 proteins in *S. cerevisiae* [65] and 32 proteins in *Arabidopsis thaliana* [66] encoded by the mtDNA, a further role played by Ymc2p and BOU is to catalyze uptake of glutamate into mitochondria for intramitochondrial protein synthesis. Finally, it is noteworthy to mention that when glutamate is generated intramitochondrially, Ymc2p and BOU can operate in the reverse direction as previously suggested for mammalian GCs [67]. Indeed, it has recently been shown that in *S. cerevisiae*, grown in synthetic minimal medium supplemented with a non-fermentable carbon source and ammonia as the main nitrogen source, ammonium fixation requires export of glutamate from the mitochondria [68]. Further experimentation is required to fully elucidate the physiological role(s) of the newly discovered mitochondrial glutamate transporter in yeast and in plants.

Author contributions

V.P., A.V., V.C., R.A. and R.G. performed the transport assays and the phylogenetic analysis of mitochondrial carriers; V.P., A.V., C.M.M., F.F. and A.C. carried out the genetic manipulation and phenotypic characterization of the yeast strains; all the authors were involved in the analysis of data; V.P., A.V., F.P. and L.P. were responsible for the experimental design and writing of the manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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