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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 *ymc2*Δ*agc1*Δ cells, reconstituted into liposomes, exhibited no glutamate transport at variance with wild-type, *ymc2*Δ and *agc1*Δ 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, *ymc2*Δ and *agc1*Δ strains, but not from the double mutant *ymc2*Δ*agc1*Δ 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_{20-30}[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 *Hind*III 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