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The *Saccharomyces cerevisiae* gene *YPR011c* encodes a mitochondrial transporter of adenosine 5′-phosphosulfate and 3′-phospho-adenosine 5′-phosphosulfate

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ABSTRACT

The genome of *Saccharomyces cerevisiae* contains 35 members of the mitochondrial carrier family, nearly all of which have been functionally characterized. In this study, the identification of the mitochondrial carrier for adenosine 5′-phosphosulfate (APS) is described. The corresponding gene (*YPR011c*) was overexpressed in bacteria. The purified protein was reconstituted into phospholipid vesicles and its transport properties and kinetic parameters were characterized. It transported APS, 3′-phospho-adenosine 5′-phosphosulfate, sulfate and phosphate almost exclusively by a counter-exchange mechanism. Transport was saturable and inhibited by bongkrekic acid and other inhibitors. To investigate the physiological significance of this carrier in *S. cerevisiae*, mutants were subjected to thermal shock at 45 °C in the presence of sulfate and in the absence of methionine. At 45 °C cells lacking *YPR011c*, engineered cells (in which APS is produced only in mitochondria) and more so the latter cells, in which the exit of mitochondrial APS is prevented by the absence of YPR011cp, were less thermotolerant. Moreover, at the same temperature all these cells contained less methionine and total glutathione than wild-type cells. Our results show that *S. cerevisiae* mitochondria are equipped with a transporter for APS and that YPR011cp-mediated mitochondrial transport of APS occurs in *S. cerevisiae* under thermal stress conditions.

1. Introduction

The *YPR011c* gene of *Saccharomyces cerevisiae* encodes a protein of unknown function (YPR011cp) that has been found to be localized in mitochondria [1,2]. To the best of our knowledge, the only data concerning *YPR011c* available until now have been obtained by microarray analysis of the genome-wide transcription profile of *S. cerevisiae* ([3,4]; website of Yeast Microarray Global Viewer (YMGV)). Firstly, *YPR011c* is one of the genes that were induced and its transcript level remained elevated after a shift of wild-type cells to higher temperatures; and secondly, *YPR011c* is among the genes that were induced in *S. cerevisiae* petit mutants lacking mitochondrial DNA and are involved in multiple cellular functions, such as heat shock. From the genome sequence of *S. cerevisiae*, it is known that *YPR011c* is located on chromosome 16 and encodes a protein with a sequence containing the characteristic features of a family of intrinsic membrane proteins, the mitochondrial carrier family (MCF) [5,6]. Thus, the primary structure of YPR011cp exhibits a tripartite structure (three repeats of about 100 amino acids), two transmembrane α-helices separated by hydrophilic loops in each repeat, and a signature motif at the C-terminus of the first helix in each repeat. The *S. cerevisiae* genome harbors 35 genes coding for members of the MCF, one of which is localized in peroxisomes and not in mitochondria [7,8]. The great majority of yeast mitochondrial carriers has been functionally characterized and shown to catalyze the transport of specific inorganic anions, metabolites, nucleotides and coenzymes across the mitochondrial or peroxisomal membrane [9–12]. By contrast, the substrate(s) transported by YPR011cp have not yet been discovered and its physiological function is yet unknown.

In the present study we report the functional characterization of the *YPR011c* protein. YPR011cp was overexpressed in *Escherichia coli*, purified, reconstituted into liposomes and identified by its transport properties and kinetic characteristics as a carrier for adenosine 5′-phosphosulfate (APS). The thermotolerance at 45 °C of *S. cerevisiae* cells lacking the gene for this carrier and of other mutants, as well as the quantification of methionine and total glutathione in these mutants and wild-type cells, provide evidence that YPR011cp transports APS from mitochondria to the cytosol under conditions of thermal stress.

Abbreviations: ApppA, P^1,P^3-di(adenosine-5′) triphosphate; APS, adenosine 5′-phosphosulfate; CoA, coenzyme A; MCF, mitochondrial carrier family; mtMet3p, mitochondrial Met3p; PAP, adenosine 3′,5′-diphosphate; PAPS, 3′-phospho-adenosine 5′-phosphosulfate; YPR011cp, the protein encoded by *YPR011c*

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2. Materials and methods

2.1. Materials

Radioactive compounds were purchased from Scopus Research. APS and PAPS were obtained from Sigma-Aldrich. Care was used in handling PAPS because it decomposes rapidly when exposed to room temperature.

2.2. Sequence search and analysis

Protein and genomic databases (www.ncbi.nlm.nih.gov) were screened with the protein sequence of *YPR011c* using BLASTP and TBLASTN.

2.3. Construction of expression plasmids

The coding sequence of *YPR011c* was amplified by PCR from *S. cerevisiae* genomic DNA, using primers corresponding to the extremities of the coding sequence with additional *Bam*HI and *Hind*III sites. For expression in *E. coli* the amplified product of *YPR011c* was cloned into the pQE30 vector (Qiagen). For expression in *S. cerevisiae* the plasmid *YPR011c*–pYES2 was constructed by cloning *YPR011c* into the pYES2/CT vector (Invitrogen) under the control of the GAL1 promoter. The plasmids *SLC25A42*–pYES2 and *LEU5*–pRS416 were prepared as described in Fiermonte et al. [13]. The plasmid Su9(1–69)–*MET3*–pYES2 was constructed by cloning the *MET3* ORF and a DNA fragment encoding the first 69 amino acids of the *Neurospora crassa* Fo ATPase subunit 9 in frame with the *MET3* ORF into the pYES2/CT vector (Invitrogen) under the control of the GAL1 promoter [14]. The *MET3* open reading frame (ORF) (*YJR010w*) was amplified from *S. cerevisiae* genomic DNA with additional *Bam*HI and *Xba*I sites. The Fo ATPase fragment was amplified from the pVT100U-GFP vector [15] with additional *Hind*III and *Bam*HI sites. The inserts of the plasmids *LEU5*–pRS416, *YPR011c*–pYES2 and *SLC25A42*–pYES2 contained an oligonucleotide, corresponding to the GKPIPNPLLGLDST peptide of the P/V proteins of the Paramyxovirus SV5, before the stop codon. All the plasmids, prepared as above, were transformed into *E. coli* DH5α cells, selected on ampicillin (100 μg/ml), and screened by direct colony PCR and by restriction digestion of purified plasmids. The sequences of the inserts were verified.

2.4. Yeast strains, media and growth conditions

BY4742 (wild-type), *YPR011c*Δ and *MET3*Δ yeast strains were provided by the EUROFAN resource center EUROSCARF (Frankfurt, Germany). The *YPR011c* or *MET3* (*YJR010w*) locus of the *S. cerevisiae* strain BY4742 (MATα; *his*3Δ1; *leu*2Δ0; l*ys*2Δ0; *ura*3Δ0) was replaced by the gentamicin resistance cassette (kanMX4). The double-deletion strain *YPR011c*Δ*MET3*Δ was constructed using the PCR-mediated gene disruption technique by replacing the *MET3* ORF with the nourseothricin resistance cassette (Nat1) in the *YPR011c*Δ mutant [16]. All deletions were verified by PCR. The *MET3*Δ–mtMet3p strain was generated by transforming *MET3*Δ with plasmid Su9(1–69)–*MET3*–pYES2 and the *YPR011c*Δ*MET3*Δ–mtMet3p strain by transforming *YPR011c*Δ*MET3*Δ with the same plasmid. The wild-type and deletion strains were grown at 30 °C in synthetic complete (SC) medium [17] supplemented with 2% glucose, 2% ethanol or 3% glycerol at pH 4.5. Transformants with the pYES2/CT plasmids were selected on the same medium without uracil.

2.5. Bacterial expression and purification of recombinant YPR011cp

YPR011cp was produced as inclusion bodies in the cytosol of *E. coli* as previously described [18], except that the host cells employed were *E. coli* M15(pREP4) (Qiagen), according to the manufacturer's instructions, and the temperature was set at 37 °C. Control cultures with the empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient [18] and washed at 4 °C first with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0), then twice with a buffer containing Triton X-114 (3%, w/v), 1 mM EDTA and 10 mM PIPES pH 7.0, and finally with 10 mM PIPES, pH 7.0. The recombinant protein was solubilized in 1.6% (w/v) sarkosyl (*N*-dodecanoyl-*N*-methylglycine sodium salt). Small residues were removed by centrifugation (20,800 ×*g* for 10 min at 4 °C).

2.6. Reconstitution of YPR011cp into liposomes

The recombinant protein in sarkosyl was reconstituted into liposomes by cyclic removal of the detergent with a hydrophobic column of Amberlite beads (Fluka), as previously described [19], with some modifications. The composition of the initial mixture used for reconstitution was 55 μl of purified YPR011cp (20 μg of protein), 80 μl of 10% Triton X-114, 90 μl of 10% phospholipids in the form of sonicated liposomes [20], 10 mM substrate except where otherwise indicated, 0.8 mg of cardiolipin, 10 mM PIPES–NaOH, pH 7.0 and water to a final volume of 700 μl. After vortexing, this mixture was recycled 13-fold through an Amberlite column (3.2 × 0.5 cm) pre-equilibrated with a buffer containing 10 mM PIPES–NaOH (pH 7.0) and the substrate at the same concentration as in the starting mixture. All operations were performed at 4 °C except for the passages through Amberlite, which were carried out at room temperature.

2.7. Transport measurements

External substrate was removed from proteoliposomes on Sephadex G-75 columns pre-equilibrated with 50 mM NaCl and 10 mM PIPES–NaOH at pH 7.0 (buffer A). The eluted proteoliposomes were distributed in reaction vessels and used for transport measurements by the inhibitor-stop method [19]. Transport at 25 °C was started by adding [^{35}S]sulfate or other indicated labeled compounds to substrate-loaded proteoliposomes (exchange) or to empty proteoliposomes (uniport). In both cases, transport was terminated by addition of 30 mM pyridoxal 5′-phosphate and 20 mM bathophenanthroline, which in combination and at high concentrations inhibit the activity of several mitochondrial carriers rapidly and completely (see, for example, [13,18,21]). In controls, the inhibitors were added at the beginning together with the radioactive substrate. Finally, the external radioactivity was removed from each sample of proteoliposomes by a Sephadex G-75 column pre-equilibrated with buffer A, and the entrapped radioactivity was measured. The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity taken up by proteoliposomes after 90 s (in the initial linear range of substrate uptake). For efflux measurements, proteoliposomes containing 2 mM sulfate or phosphate were labeled with 20 μM [^{35}S] sulfate or [^{33}P]phosphate by carrier-mediated exchange equilibration [19]. After 40 min, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75 columns pre-equilibrated with buffer A. Efflux was started by adding unlabeled external substrate or buffer A alone to aliquots of proteoliposomes and terminated by adding the inhibitors indicated above.

2.8. Subcellular localization of Met3p and mtMet3p

The coding sequences for Met3p (*YJR010w*) and Su9(1–69)–Met3p (mtMet3p), amplified as described above but without termination codons, were cloned in frame with the yEGFP (yeast-enhanced green fluorescence protein) coding sequence into the pYES2/CT vector. The wild-type yeast strain BY4742 was transformed with each of the GFP fusion constructs under the control of the GAL1 promoter. Cells were grown in glycerol-supplemented SC medium to mid-logarithmic phase and transferred in 3% glycerol- and 0.4% galactose-supplemented SC medium. After 4 h the cells were incubated for 1 h at 30 °C in the presence of

50 nM MitoTracker Red CMXRos (Molecular Probes), washed twice with fresh medium, and imaged at the microscope as described previously [22,23].

2.9. Heat shock treatment

Wild-type, *YPR011c*Δ, *MET3*Δ–mtMet3p and *YPR011c*Δ*MET3*Δ–mtMet3p yeast strains were grown at 30 °C in methionine-less SC medium supplemented with 2% ethanol and 0.2% galactose. Each strain was allowed to grow to 2×10^7 cells ml^{-1} and then was transferred in a thermal incubator at 45 °C. Cell viability following 45 °C treatment was assayed by colony formation on YEPD (1% bacto-yeast extract, 2% Bacto-Peptone, 2% glucose, 2% Bacto-agar) plates. The plates were incubated at 30 °C for 2 days and then the colonies were counted [24]. Controls were grown as indicated above, except that they were maintained at 30 °C without undergoing the temperature change to 45 °C.

2.10. Methionine and glutathione determinations by mass spectrometry

Wild-type and mutant strains were grown at 30 °C in methionine-less SC medium supplemented with 2% ethanol and 0.2% galactose to 2×10^7 cells ml^{-1} and transferred at 45 °C for 1 h. The cells were then washed at 300 ×*g* for 20 min at 4 °C to remove dead cells. The pellets were accurately weighed and suspended in cold methanol, centrifuged at 20,800 ×*g* for 12 min at 4 °C and kept at −80 °C overnight. A Quattro Premier mass spectrometer coupled to an Acquity UPLC system (Waters, Milford, USA) was used for electrospray ionization LC–MS/MS analysis. Quantification was achieved through calibration curves set at five concentrations of standards processed under the same conditions as samples. The best fit was determined using regression analysis of the peak analyte area. Data were normalized to the amount of live cells for each strain. The multiple reaction monitoring (MRM) transitions monitored in the positive ion mode were *m/z* 149.1 > 104.1 for methionine, *m/z* 308.3 > 179.0 for reduced glutathione (GSH), and *m/z* 613.2 > 484.2 for oxidized glutathione (GSSG). Chromatographic separation was achieved using a BEH C18 column (2.1 × 50 mm, 1.7 μm particle size, Waters) eluted with a linear gradient from 100% (initial phase) 0.5 mM TDFHA to 100% 0.5 mM TDFHA/90% acetonitrile. The UPLC flow rate was set at 0.4 ml min^{-1}.

2.11. Other methods

Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue dye. The identity of purified YPR011cp was assessed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry of trypsin digests of the corresponding band excised from a Coomassie-stained gel [25]. The amount of pure YPR011cp was estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard [26]. To assay the protein incorporated into liposomes, the vesicles were passed through a Sephadex G-75 column, centrifuged at 300,000 ×*g* for 30 min and delipidated with organic solvents as described in Capobianco et al. [27]. Then, the SDS-solubilized protein was determined by comparison with carbonic anhydrase in SDS gels. The share of incorporated protein was about 16% of the protein added to the reconstitution mixture. The expression levels of Leu5p, YPR011cp and SLC25A42 in *S. cerevisiae LEU5*Δ cells were determined using the mouse monoclonal anti-V5 antibody (Sigma-Aldrich).

3. Results

3.1. YPR011cp overexpression does not function as a coenzyme A transporter in S. cerevisiae

The *LEU5* null mutant does not grow on YP supplemented with a nonfermentable substrate due to its inability to import coenzyme A (CoA) into the mitochondrial matrix [28]. The fact that the endogenously present YPR011cp does not prevent the severe growth defect of the LEU5Δ knock-out suggests that YPR011cp is unable to transport CoA into mitochondria. However, in a phylogenetic tree of all *S. cerevisiae*, *Homo sapiens* and *Arabidopsis thaliana* MCF proteins [5] YPR011cp clusters together with the human, yeast and Arabidopsis transporters for coenzyme A (SLC25A42, LEU5p, At1g14560p and At4g26180p, respectively) [13,28,29] and some other mitochondrial carriers whose function has not yet been identified. Furthermore, BLAST searches of the human, yeast and Arabidopsis genomes using the YPR011cp sequence as query detected SLC25A42, LEU5p, At1g14560p and At4g26180p as the closest relatives of YPR011cp, although the percentage of identical amino acids they share is not much higher than the basic homology existing between the different members of the MCF (29% between YPR011cp and SLC25A42, 31% between YPR011cp and LEU5p, and 32–34% between YPR011cp and At4g26180p and At1g14560p). In view of the above bioinformatics data we tested whether overexpression of YPR011cp mitigates or abolishes the severe growth defect of the *LEU5*Δ knockout. Fig. 1 shows that YPR011cp expressed in *LEU5*Δ cells via the multicopy yeast vector pYES2 did not restore growth of the *LEU5*Δ strain on glycerol. By contrast, when the *LEU5*Δ cells were transformed with the same vector carrying the coding sequence of SLC25A42 or with the pRS416 plasmid carrying the coding sequence of LEU5p, growth was restored almost to wild-type levels. The lack of complementation of *LEU5*Δ by YPR011cp overexpression confirms the conjecture that YPR011cp cannot import CoA into mitochondria.

3.2. Bacterial expression of YPR011cp

The *YPR011c* gene was expressed in *E. coli* M15 (pREP4) (Supplementary Fig. S1, lane 4). The gene product accumulated as inclusion bodies and was purified by centrifugation and washing. The apparent molecular mass of the purified protein was 42.3 kDa (Supplementary Fig. S1, lane 5). Its identity was confirmed by MALDI-TOF mass spectrometry, and the yield of the purified protein was approximately 30 mg per liter of culture. The protein was not detected in bacteria harvested immediately before induction of expression (Supplementary Fig. S1, lane 2) nor in cells harvested after induction but lacking the coding sequence in the expression vector (Supplementary Fig. S1, lane 3).

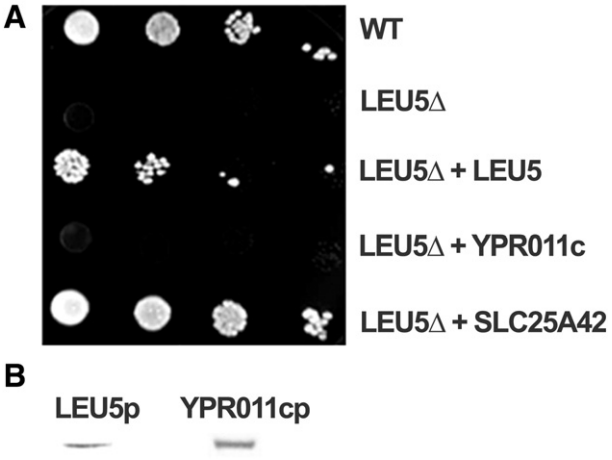

Fig. 1. Effect of complementing *LEU5*Δ cells with YPR011cp and SLC25A42 on growth. (A) Four-fold serial dilutions of wild-type cells (WT), *LEU5*Δ cells and *LEU5*Δ cells transformed with the *YPR011c*–pYES2, *SLC25A42*–pYES2, or *LEU5*–pRS416 plasmid (LEU5Δ + YPR011c, LEU5Δ + SLC25A42, or LEU5Δ + LEU5, respectively) were plated on YP medium supplemented with 3% glycerol and 0.2% galactose and incubated for 72 h at 30 °C. (B) Expression levels of Leu5p and YPR011cp in *LEU5*Δ cells. Mitochondrial lysates (30 μg of protein) from *LEU5*Δ cells transformed with the *LEU5*–pRS416 plasmid (LEU5Δ + LEU5) or the *YPR011c*–pYES2 plasmid (LEU5Δ + YPR011c), grown in YP medium supplemented with 2% glucose, were separated by SDS-PAGE, transferred to nitrocellulose and immunodecorated with the monoclonal anti-V5 antibody.

3.3. Functional characterization of recombinant YPR011cp

In the search for potential substrates of YPR011cp, the recombinant purified YPR011cp was reconstituted into liposomes and its transport activity was tested in homo-exchange experiments (i.e., with the same substrate internally and externally). Using internal and external substrate concentrations of 10 and 0.2 mM, respectively, the reconstituted protein catalyzed active [^{35}S]sulfate/sulfate and [^{33}P]phosphate/phosphate exchanges that were inhibited completely by a mixture of pyridoxal 5′-phosphate and bathophenanthroline. In contrast, despite the long incubation period (i.e., 30 min) very little homo-exchange activity was observed for AMP, ADP, ATP, GTP, UTP, CDP and CTP (Fig. 2). Furthermore, no [^{35}S]sulfate/sulfate and [^{33}P]phosphate/phosphate exchange activities were detected if YPR011cp had been boiled before incorporation into liposomes or if proteoliposomes were reconstituted with sarkosyl-solubilized material from bacterial cells either lacking the expression vector for YPR011cp or harvested immediately before induction of expression.

To investigate the substrate specificity of YPR011cp further, the initial rate of [^{35}S]sulfate uptake into proteoliposomes that had been preloaded with various potential substrates was measured (Fig. 3). The highest activities of sulfate uptake into proteoliposomes were found with internal sulfate, thiosulfate, pyrosulfate, phosphate, pyrophosphate, adenosine 5′-phosphosulfate (APS) and 3′-phospho-adenosine 5′-phosphosulfate (PAPS). In contrast, a marginal exchange of [^{35}S]sulfate was observed with internal AMP, ADP, ATP, ATP-Mg, dAMP, dADP, dATP, 3′-AMP, cAMP, P^1,P^3-di(adenosine-5′) triphosphate (ApppA), CoA, dephospho-CoA, adenosine 3′,5′-diphosphate (PAP), *S*-adenosylmethionine, NAD$^+$, NMN, FAD, FMN, ThMP, ThPP, methionine, cysteine, glutathione, glutamate, arginine, NaCl (Fig. 3), and thiamine, succinate, malate, oxoglutarate, citrate, carnitine, aspartate, glutamine, lysine, histidine and proline (data not shown). Similar results of Fig. 3 were obtained using [^{33}P]phosphate instead of [^{35}S]sulfate (data not shown).

Given that CoA, dephospho-CoA and PAP are transported by SLC25A42 [13] the closest human relative of YPR011cp, the ability of YPR011cp to transport these compounds was further investigated. However, no YPR011cp-catalyzed activity of CoA, dephospho-CoA or PAP transport in exchange with sulfate or phosphate was detected under any of the experimental conditions tested, which included variation of the parameters that influence solubilization of inclusion bodies, reconstitution into liposomes and transport assay. Moreover,

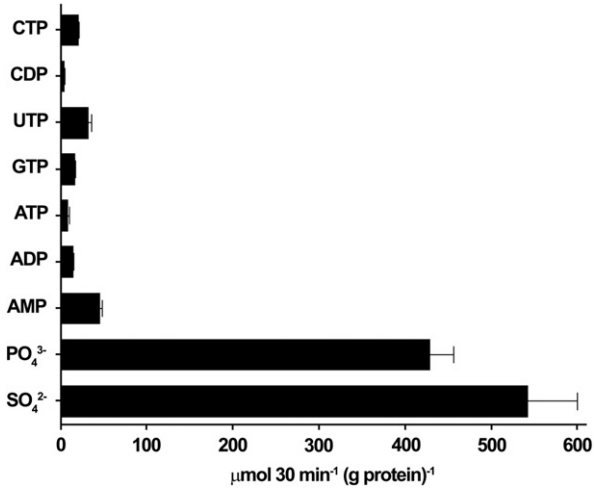

Fig. 2. Homo-exchanges of sulfate, phosphate and nucleotides in proteoliposomes containing recombinant YPR011cp. Transport was initiated by adding radioactive substrate (final concentration, 0.2 mM) to proteoliposomes preloaded internally with the same substrate (concentration, 10 mM). The reaction was terminated after 30 min. Values are the means ± S.D. of at least three independent experiments.

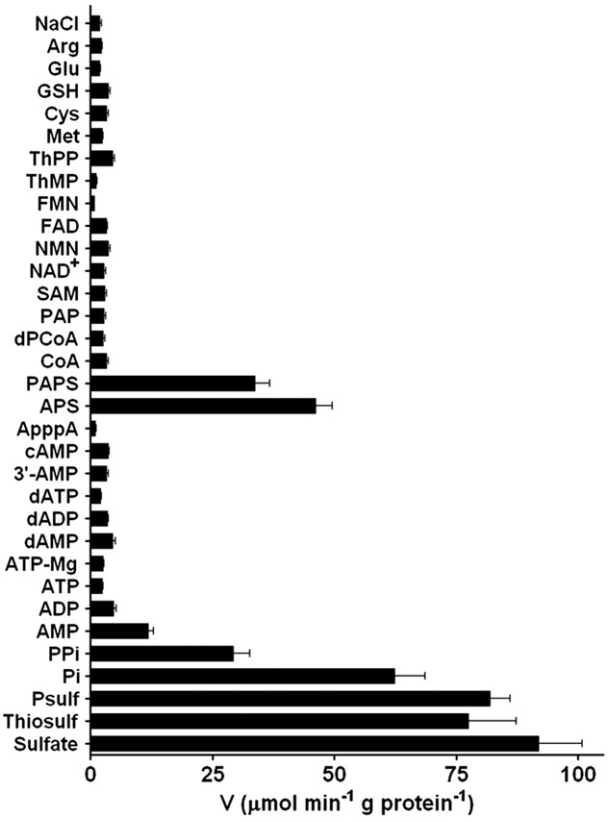

Fig. 3. Substrate specificity of YPR011cp. Liposomes reconstituted with YPR011cp were preloaded internally with various substrates (concentration, 10 mM). Transport was started by adding 0.32 mM of [^{35}S]sulfate and terminated after 90 s. Values are means ± S.D. of at least three independent experiments. Abbreviations: Thiosulf, thiosulfate; Psulf, pyrosulfate; PPi, pyrophosphate; ApppA, P^1,P^3-di(adenosine-5′) triphosphate; APS, adenosine 5′-phosphosulfate; PAPS, 3′-phospho-adenosine 5′-phosphosulfate; CoA, coenzyme A; dPCoA, dephospho-CoA; PAP, adenosine 3′,5′-diphosphate; SAM, *S*-adenosylmethionine; ThMP, thiamine monophosphate; ThPP, thiamine pyrophosphate; Met, methionine; Cys, cysteine; GSH, reduced glutathione; Glu, glutamate; Arg, arginine.

YPR011cp did not transport the above-mentioned compounds under the same experimental conditions employed for measuring the activity of SLC25A42 [13].

The [^{35}S]sulfate/sulfate exchange reaction catalyzed by reconstituted YPR011cp was inhibited by externally added unlabeled APS, PAPS, sulfate and phosphate in a concentration-dependent manner (Fig. 4). At concentrations lower than 1 mM APS and PAPS displayed a much higher inhibitory potency than sulfate and phosphate, in agreement with their respective inhibition constants (see below). The effects of inhibitors of other mitochondrial carriers on the YPR011cp-mediated [^{35}S]sulfate/sulfate exchange were also examined (Supplementary Fig. S2). This exchange was inhibited strongly by pyridoxal 5′-phosphate, bathophenanthroline, mercurials (mercuric chloride, *p*-hydroxymercuribenzoate and mersalyl), as well as by tannic acid and bromocresol purple and poorly by *N*-ethylmaleimide and α-cyano-4-hydroxycinnamate. Interestingly, bongkrekic acid inhibited the activity of YPR011cp partially (53%), whereas carboxyatractyloside had very little effect (11%) at a concentration (10 μM) that completely inhibits the ADP/ATP carrier [30].

3.4. Kinetic characteristics of recombinant YPR011cp

The kinetics were compared for the uptake of 1 mM [^{35}S]sulfate or [^{33}P]phosphate into proteoliposomes either as uniport (in the absence of internal substrate) or as exchange (in the presence of 10 mM sulfate, phosphate or APS) (Fig. 5A and B). Both sulfate/sulfate and phosphate/phosphate exchanges followed a first-order

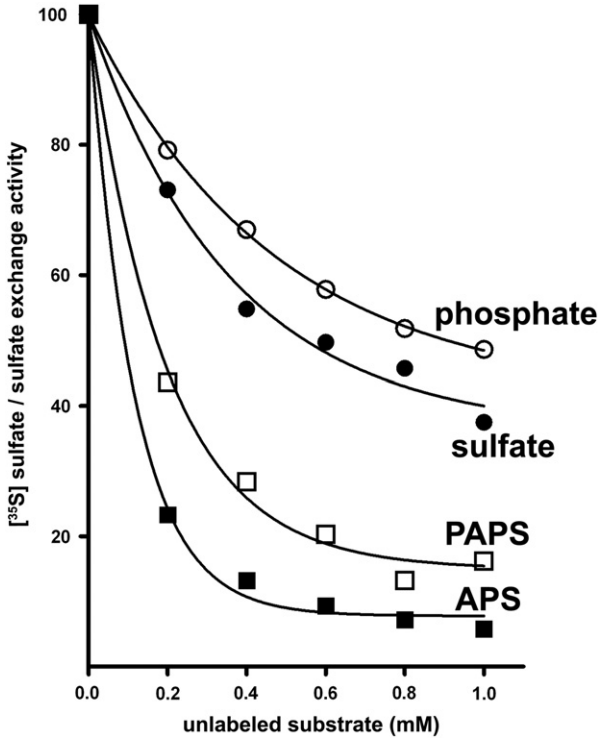

Fig. 4. Effect of unlabeled substrates on the [^{35}S]sulfate/sulfate exchange by YPR011cp. Liposomes were reconstituted with YPR011cp and preloaded internally with 10 mM sulfate. Transport was initiated by adding 0.32 mM [^{35}S]sulfate to proteoliposomes and terminated after 90 s. Where indicated, increasing concentrations of unlabeled phosphate, sulfate, APS and PAPS were added simultaneously with [^{35}S]sulfate.

kinetics (rate constants, 0.13 and 0.12 min^{-1}; initial rates, 145 and 121 μmol min^{-1} g $protein^{-1}$, respectively), with isotopic equilibrium being approached exponentially. The uptakes of [^{35}S]sulfate and [^{33}P]phosphate in exchange with internal APS also followed a first-order kinetics, but their initial rates were lower because the Vmax values of the [^{35}S]sulfate/APS and [^{33}P]phosphate/APS exchanges are considerably lower than those of sulfate/sulfate and phosphate/phosphate exchanges (see below). In contrast, the uniport uptake of sulfate or phosphate was negligible. The uniport mode of transport by YPR011cp was further investigated by measuring the efflux of [^{35}S]sulfate and [^{33}P]phosphate from preloaded proteoliposomes, as this provides a more sensitive assay for unidirectional transport [19]. In these experiments, little efflux of [^{35}S]sulfate or [^{33}P]phosphate from prelabeled proteoliposomes was observed with the addition of buffer alone, i.e. in the absence of external substrate (Fig. 5C and D). However, extensive efflux occurred upon addition of external sulfate, phosphate or APS (Fig. 5C and D). Both effluxes, i.e. with and without external substrate, were prevented completely if the inhibitors pyridoxal 5′-phosphate and bathophenanthroline were present. Therefore, YPR011cp is able to catalyze a fast exchange reaction of substrates and only a very slow unidirectional transport of sulfate or phosphate. Furthermore, the addition of 5 mM CoA, dephospho-CoA or PAP to prelabeled proteoliposomes did not induce any increase in sulfate or phosphate efflux as compared to the very small effluxes observed by adding buffer alone (results not shown), indicating that CoA, dephospho-CoA and PAP are not transported by YPR011cp.

The kinetic constants of reconstituted YPR011cp were determined by measuring the initial transport rate at various external [^{35}S]sulfate or [^{33}P]phosphate concentrations in the presence of a fixed saturating concentration (10 mM) of sulfate, phosphate, APS and PAPS. Whereas the transport affinities (Km) for sulfate and phosphate (about 330 and

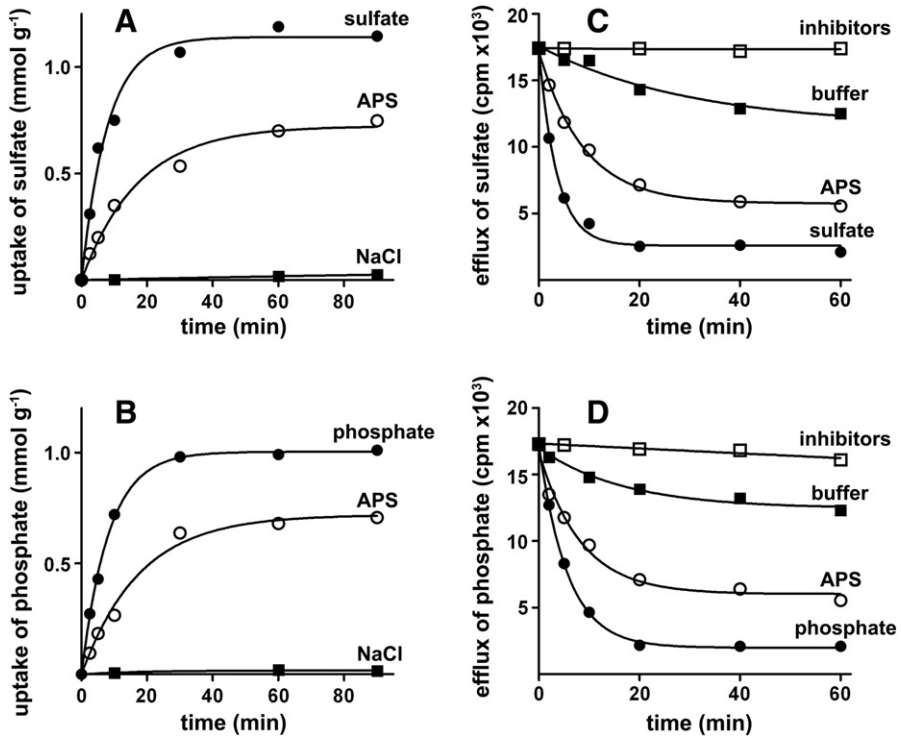

Fig. 5. Kinetics of [^{35}S]sulfate or [^{33}P]phosphate transport in proteoliposomes reconstituted with YPR011cp. (A) and (B), uptake of sulfate and phosphate. 1 mM [^{35}S]sulfate (A) or 1 mM [^{33}P]phosphate (B) was added to proteoliposomes containing 10 mM sulfate or phosphate (●), 10 mM APS (○) or 10 mM NaCl and no substrate (uniport, (■)). (C) and (D), efflux of [^{35}S]sulfate or [^{33}P]phosphate from proteoliposomes reconstituted with YPR011cp. The internal substrate of proteoliposomes (2 mM sulfate (C) or 2 mM phosphate (D)) was labeled with [^{35}S]sulfate (C) or [^{33}P]phosphate (D) by carrier-mediated exchange equilibration. After removal of the external substrate by Sephadex G-75, the efflux of [^{35}S]sulfate (C) or [^{33}P]phosphate (D) was started by adding buffer A alone (■), 5 mM sulfate or phosphate in buffer A (●), 5 mM APS in buffer A (○), or 5 mM sulfate or phosphate, 30 mM pyridoxal 5′-phosphate and 20 mM bathophenanthroline in buffer A (□). Similar results were obtained in three independent experiments.

480 μM, respectively) were not significantly different for all the exchanges tested, the Vmax values for both the homo- and hetero-exchanges of sulfate and phosphate were substantially higher ($P < 0.01$) than those for the hetero-exchanges between sulfate or phosphate and APS or PAPS (Table 1). Several external substrates were competitive inhibitors of [^{35}S]sulfate uptake as they increased the apparent Km without changing the Vmax (not shown). The inhibitory constants (Ki) of these compounds for YPR011cp are the following: 38 ± 5 μM (APS), 96 ± 13 μM (PAPS), 343 ± 28 μM (sulfate), 420 ± 49 μM (phosphate), 218 ± 34 μM (thiosulfate), 439 ± 37 μM (CoA), 532 ± 47 μM (PAP), and 692 ± 55 μM (AMP). These results show that APS and PAPS are the highest affinity external substrates. The Ki of CoA, PAP and AMP are significantly higher ($P < 0.01$) than those of the other compounds listed above; these data show that CoA, PAP and AMP, though not transported by YPR011cp, can react with this carrier in agreement with the fact that YPR011cp and the *S. cerevisiae*, plant and human CoA transporters cluster together [5].

3.5. Thermotolerance is reduced in YPR011cΔ and other mutants

Having established the transport function of YPR011cp in vitro, we investigated the physiological significance of *YPR011c* in yeast cells. APS and PAPS, the best substrates of YPR011cp, are synthesized by the enzymes ATP sulfurylase and adenylylsulfate kinase (encoded by the *MET3* and *MET14* genes, respectively) that control the first steps of the sulfur assimilation pathway leading to the production of methionine, cysteine and glutathione [31]. Furthermore, APS and PAPS are responsible for the thermotolerance of *S. cerevisiae* cells in the absence of methionine [24]; in the presence of methionine *S. cerevisiae* cells lose viability after sudden elevation of temperature from 30 °C to 45 °C due to the repression of *MET3* and *MET14* by methionine and the consequent lack of APS and PAPS production [24]. Given that ATP sulfurylase is localized both in the cytosol and in mitochondria [1,2,32], we set out to investigate the contribution of mitochondrial and cytosolic APS to *S. cerevisiae* thermotolerance. For this purpose, the *S. cerevisiae* mutant strains *YPR011c*Δ, *MET3*Δ–*mtMet3p* and *YPR011c*Δ*MET3*Δ*mtMet3p* were employed. The *MET3*Δ–*mtMet3p* and *YPR011c*Δ*MET3*Δ*mtMet3p* yeast strains were produced by transforming *MET3*Δ and the double mutant *YPR011c*Δ*MET3*Δ with the plasmid Su9(1–69)–Met3p (see Materials and methods). Supplementary Fig. 3B shows that Su9(1–69)–Met3p (i.e., mtMet3p) fused with GFP localized exclusively to the mitochondria. Thus, the fluorescence of GFP-fused mtMet3p completely overlapped the red fluorescence displayed by the mitochondrial-specific dye, MitoTracker Red, whereas *S. cerevisiae* cells expressing Met3p fused to GFP showed a widely diffuse green fluorescence (Supplementary Fig. 3A). In view of the substrate specificity of YPR011cp it is predicted that *i)* in the *YPR011c*Δ cells APS is produced in mitochondria and cytosol but its transport between these compartments is blocked, *ii)* in the *MET3*Δ–*mtMet3p* cells APS is synthesized only in mitochondria and can be transported to the cytosol by YPR011cp, and *iii)* in *YPR011c*Δ*MET3*Δ–mtMet3p APS is synthesized only in mitochondria but its efflux from these organelles is prevented by the absence of YPR011cp. These *S. cerevisiae* mutant and wild-type cells had been left to grow at 30 °C in methionine-less, ethanol-supplemented SC medium to 2 × 10^7 cells ml^{-1} before being transferred in a thermal incubator at 45 °C to compare their viability after the indicated heat shock time periods (Fig. 6). Upon thermal shift to 45 °C the *YPR011c*Δ and *MET3*Δ–mtMet3p cells displayed less thermotolerance than wild-type cells, and the *YPR011c*Δ*MET3*Δ–mtMet3p cells lost viability more rapidly than the single mutants, approaching the death rate of the wild-type cells in the presence of methionine.

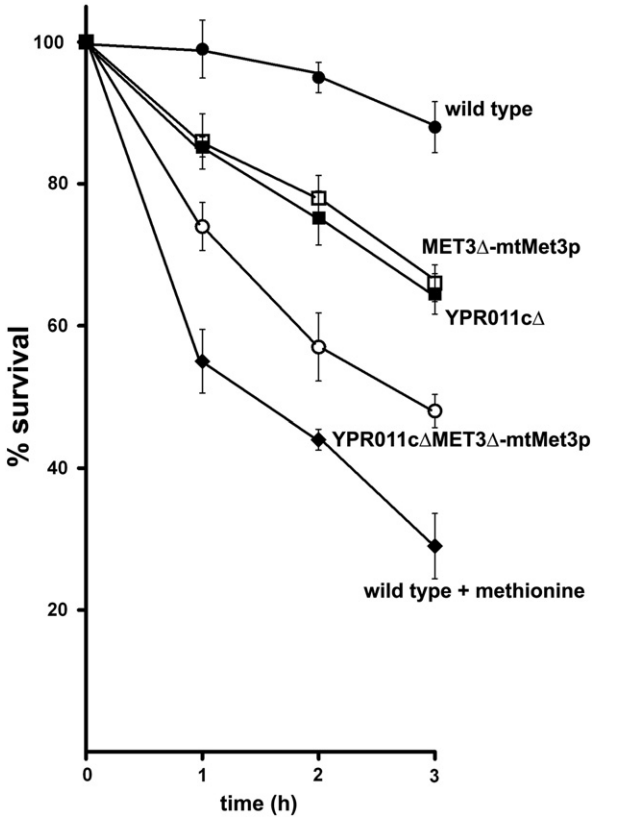

Fig. 6. Thermotolerance of wild-type, *YPR011c*(δ)*MET3*(δ)–mtMet3p and *YPR011c*(δ)*MET3*(δ)–mtMet3p cells shifted from 30 to 45 °C. Wild-type and deletion strains were pre-incubated in glucose-supplemented SC medium until the stationary phase was reached and washed three times with methionine-less SC medium. Then wild-type (●), *YPR011c*Δ ■, *MET3*Δ–mtMet3p (□) and *YPR011c*Δ*MET3*Δ–mtMet3p (○) cells were grown at 30 °C in methionine-less SC medium supplemented with 2% ethanol and 0.2% galactose to 2 × 10^7 cells ml^{-1} and shifted to 45 °C. Wild-type cells were also treated as above except that the medium was supplemented with 3 mM methionine (♦). At the indicated times after the temperature shift to 45 °C the same portion of each culture was diluted in distilled water and immediately spread onto solid YEPD.

Table 1
Kinetic parameters of reconstituted YPR011cp. Km and Vmax values were calculated from double-reciprocal plots of the rates of [^{35}S]sulfate or [^{33}P]phosphate uptake at various external sulfate or phosphate concentrations into liposomes reconstituted with YPR011cp and containing the indicated substrates (10 mM). The data represent the means ± S.D. of at least four independent experiments.

External substrate	Internal substrate	Vmax (μmol min^{-1} g protein^{-1})	Km (μM)
[^{35}S]sulfate	Sulfate	193 ± 26	327 ± 28
[^{35}S]sulfate	Phosphate	181 ± 30	350 ± 37
[^{35}S]sulfate	APS	90 ± 12	317 ± 29
[^{35}S]sulfate	PAPS	88 ± 16	344 ± 34
[^{33}P]phosphate	Phosphate	168 ± 25	508 ± 57
[^{33}P]phosphate	Sulfate	185 ± 21	455 ± 43
[^{33}P]phosphate	APS	96 ± 16	477 ± 50
[^{33}P]phosphate	PAPS	81 ± 11	486 ± 56

3.6. YPR011cΔ and other mutants contain less methionine and glutathione than wild-type at high temperatures

To gain further insight into the physiological role of the *YPR011c* gene product, cellular levels of metabolites derived from APS and PAPS were assessed after a 1-h thermal shift from 30 to 45 °C of wild-type, *YPR011c*Δ, *MET3*Δ-mtMet3p and *YPR011c*Δ*MET3*Δ-mtMet3p cells grown in methionine-less, ethanol-supplemented SC medium. At 45 °C cellular methionine levels were significantly reduced in the *YPR011c*Δ and *MET3*Δ-mtMet3p cells as compared to wild-type (Fig. 7A). Furthermore, the amount of methionine in the *YPR011c*Δ*MET3*Δ-mtMet3p cells was significantly less than in the *YPR011c*Δ and *MET3*Δ-mtMet3p cells, and about 6 times less than in wild-type cells. Similarly, total glutathione levels (GSH + GSSG) at 45 °C

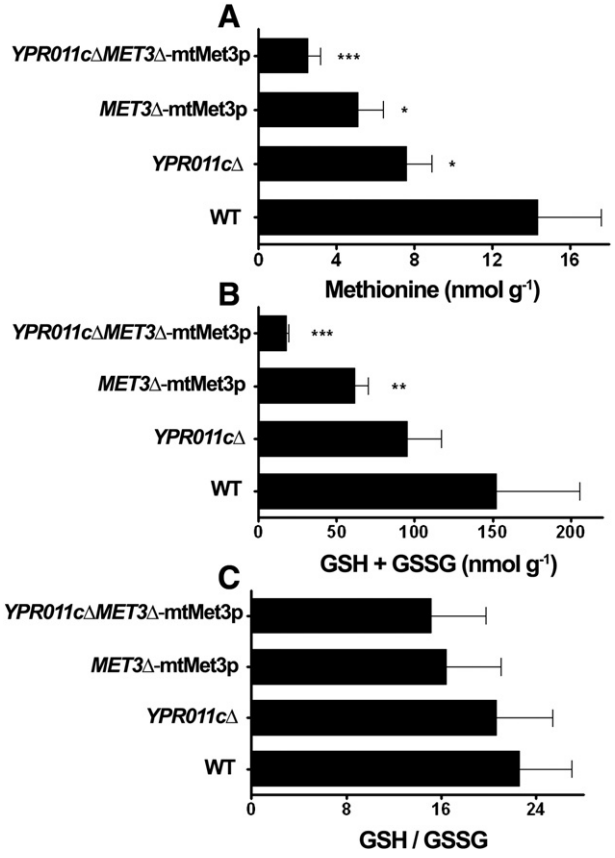

Fig. 7. Levels of methionine and total glutathione and GSH/GSSG ratios in wild-type, *YPR011c*Δ, *MET3*Δ–mtMet3p and *YPR011c*Δ*MET3*Δ–Met3p cells at 45 °C. Yeast strains were pre-incubated in glucose-supplemented SC medium until the stationary phase was reached and washed three times with methionine-less SC medium. Then they were grown at 30 °C in methionine-less SC medium supplemented with 2% ethanol and 0.2% galactose to 2×10^7 cells ml^{-1} and shifted at 45 °C for 1 h. Cellular extracts were assayed for (A) methionine content, (B) the sum of GSH + GSSG levels and (C) the GSH/GSSG ratios by LC–MS/MS analysis. Means ± S.E.M. of at least four independent experiments are reported. Differences between methionine of *YPR011c*Δ, *MET3*Δ–mtMet3p and control (wild-type [WT]) cells were significant (*$P < 0.05$). Differences between methionine of *YPR011c*Δ*MET3*Δ–mtMet3p and *YPR011c*Δ, *MET3*Δ–mtMet3p and control cells (WT) were significant (***$P < 0.05$). Differences between the sum of GSH + GSSG of *MET3*Δ–mtMet3p and both *YPR011c*Δ and control (WT) cells were significant (**$P < 0.05$). Differences between the sum of GSH + GSSG of *YPR011c*Δ*MET3*Δ–mtMet3p and *MET3*Δ–mtMet3p, *YPR011c*Δ, and control (WT) cells were significant (***$P < 0.05$). Differences between the GSH/GSSG ratios of *YPR011c*Δ, *MET3*Δ–mtMet3p and *YPR011c*Δ*MET3*Δ–mtMet3p and control (WT) cells were not significant ($P > 0.05$).

were reduced to about 62% in *YPR011c*Δ, 30% in *MET3Δ*-mtMet3p and 10% in *YPR011c*Δ–mtMet3p cells compared to wild-type, with no significant difference in the GSH/GSSG ratio (Fig. 7B and C). The cellular levels of cysteine after 1-h at 45 °C did not display any notable difference among all the strains investigated (data not shown). These results indicate that the mitochondrial carrier YPR011cp, under the experimental conditions employed, is involved in the production of methionine and glutathione.

4. Discussion

The results presented in this study, together with those concerning YPR011cp targeting to mitochondria [1,2], demonstrate that YPR011cp is a mitochondrial transporter for APS and PAPS. Besides transporting APS and PAPS, recombinant and reconstituted YPR011cp also transports sulfate, phosphate, thiosulfate and pyrosulfate. Among purine nucleotides, which are structurally very closely related to APS and PAPS, only AMP is transported to a very low extent. Additional nucleotides, dinucleotides and many other compounds tested are not transported by YPR011cp. The affinities of YPR011cp for APS and PAPS are higher than those for sulfate and phosphate. However, the Vmax values for APS and PAPS are lower than those for sulfate and phosphate. In this respect, YPR011cp differs from many characterized mitochondrial carriers that exhibit almost identical Vmax values for all the transported substrates [12,33–41]. The known exception is represented by SLC25A42, which exhibits significantly lower Vmax values for CoA and dephospho-CoA than for ADP and PAP [13]. Presumably, the Vmax values of YPR011cp and SLC25A42 (and of other MCF members of the same cluster [5]) vary with substrate size.

The substrate specificity of YPR011cp is distinct from that of any other previously characterized member of the MCF. In particular, YPR011cp differs from the members of the MCF which it clusters together with and whose functions are known: SLC25A42, LEU5p, At1g14560p and At4g26180p because YPR011cp does not transport CoA, dephospho-CoA, PAP, ADP and ATP [13,28,29]; At4g01100 (ADT1), which transports ATP, ADP and AMP as the best substrates [42]; and At5g01500, which has been recently identified as a transporter of PAPS from chloroplasts to the cytosol in exchange for PAP [43]. YPR011cp is also different from the animal Golgi-resident PAPS transporters, which are all members of the nucleotide-sugar transporter family [44,45]. Because YPR011cp functions almost exclusively by a counter-exchange mechanism, our transport measurements in the reconstituted system indicate that APS and PAPS may cross the mitochondrial membrane in both directions via YPR011cp in exchange with sulfate or phosphate (which are also transported by other mitochondrial carriers [9]). However, this holds true only for APS, which is produced by the *MET3* gene product, ATP sulfurylase, that has a dual cellular localization—cytosolic and mitochondrial [1,2,32]. PAPS, however, is produced only in the cytosol by adenylylsulfate kinase, which phosphorylates APS to PAPS.

S. cerevisiae cells do not survive in the absence of APS and PAPS upon a temperature shift from 30 to 45 °C [24], although the mechanism of thermal protection by these sulfur nucleotides is elusive. Under the same conditions of thermal stress used by Jakubowski and Goldman [24], i.e. in the presence of sulfate and absence of methionine, our results obtained by subjecting various *S. cerevisiae* mutants to thermal shock strongly suggest that APS produced in mitochondria is transported from the mitochondrial matrix to the cytosol via YPR011cp. In fact, the *YPR011c*Δ*MET3*Δ–mtMet3p strain (in which APS originates only in mitochondria but its efflux is prevented by the absence of YPR011cp) is more susceptible to thermal stress than the *MET3*Δ–mtMet3p strain (in which APS synthesis is solely mitochondrial and APS can be transported to the cytosol). Furthermore, the observation that the *YPR011c*Δ strain (in which the APS produced in mitochondria cannot exit the organelles) is susceptible to thermal shift demonstrates that both cytosolic and mitochondrial APS are crucial to support *S. cerevisiae* cell survival at high temperatures.

Given that all the enzymes of the sulfur assimilation pathway required for methionine and glutathione production are localized in the cytosol (except for the dually located ATP sulfurylase) and that glutathione increases under thermal stress [46,47] and is produced, at least partly, by APS, the APS demand in yeast cells at 45 °C could be greater and the contribution of mitochondrial APS necessary. The methionine and total glutathione levels in *YPR011c*Δ cells, as well as in *MET3*Δ–mtMet3p cells, compared to those of wild-type cells support this hypothesis. The presence of a smaller amount of methionine and total glutathione in the *MET3*Δ–mtMet3p cells compared to *YPR011c*Δ cells can be explained by a lower supply of APS from mitochondria than from the cytosol. Nevertheless, the importance of both mitochondrial and cytosolic APS in the synthesis of glutathione and methionine at 45 °C is demonstrated by the finding that total glutathione and methionine levels are still lower in the *YPR011c*Δ*MET3*Δ–mtMet3p cells as compared to the *YPR011c*Δ and *MET3*Δ–mtMet3p cells. Taken all together the results reported above show that yeast cells grown in the absence of methionine respond to conditions of thermal stress by exporting APS from mitochondria to the cytosol via YPR011cp. Our

data, and in particular the quantification of glutathione and methionine, also suggest that APS-mediated thermal protection may be, at least in part, related to the synthesis of glutathione which is necessary for protecting cells at high temperatures [46] and for replenishing cells with sulfur metabolites. Another possible role of APS in thermotolerance is its ability to produce PAPS for the sulfation of key temperature-sensitive proteins, although sulfation of proteins or other compounds has never been demonstrated in *S. cerevisiae* until now. Moreover, APS could be used for the biosynthesis of AppppA (P^1,P^4-di(adenosine-5′) tetraphosphate) [48], an unusual dinucleotide polyphosphate known to markedly increase in yeast after hyperthermic treatment [49,50]. This is the first time that evidence is provided for the participation of mitochondria in thermotolerance and specifically by supplying APS to the cytosol, which may trigger a signaling mechanism that is crucial for cell survival at higher temperatures.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.11.013.

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