THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 3, Issue of January 19, pp. 1916–1922, 2001

Printed in U.S.A.

Identification in *Saccharomyces cerevisiae* of Two Isoforms of a Novel Mitochondrial Transporter for 2-Oxoadipate and 2-Oxoglutarate*

Received for publication, May 19, 2000, and in revised form, September 25, 2000
Published, JBC Papers in Press, September 29, 2000, DOI 10.1074/jbc.M004332200

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The nuclear genome of *Saccharomyces cerevisiae* encodes 35 members of a family of membrane proteins. Known members transport substrates and products across the inner membranes of mitochondria. We have localized two hitherto unidentified family members, Odc1p and Odc2p, to the inner membranes of mitochondria. They are isoforms with 61% sequence identity, and we have shown in reconstituted liposomes that they transport the oxodicarboxylates 2-oxoadipate and 2-oxoglutarate by a strict counter exchange mechanism. Intraliposomal adipate and glutarate and to a lesser extent malate and citrate supported [^{14}C]oxoglutarate uptake. The expression of Odc1p, the more abundant isoform, made in the presence of nonfermentable carbon sources, is repressed by glucose. The main physiological roles of Odc1p and Odc2p are probably to supply 2-oxoadipate and 2-oxoglutarate from the mitochondrial matrix to the cytosol where they are used in the biosynthesis of lysine and glutamate, respectively, and in lysine catabolism.

A family of membrane proteins that transports metabolites involved in oxidative phosphorylation and in other important functions in mitochondria is found in the inner membranes of the organelle (1). The sequences of members of this family are made of three related domains of about 100 amino acids repeated in tandem, each probably being folded into two transmembrane α-helices joined by an extensive hydrophilic sequence. The three repeats are linked by shorter hydrophilic sequences. The repeats in the various family members are all related, and various sequence features are conserved (2, 3). The nuclear genome of *Saccharomyces cerevisiae* encodes 35 members of this family (4). They include three isoforms of the ADP/ATP translocase and the carriers for phosphate, citrate, dicarboxylate, ornithine, succinate-fumarate, oxaloacetate-sulfate, and carnitine (see Ref. 5 for a review; Refs. 6 and 7). Hitherto, the functions of other family members have been unknown. Two of them, Odc1p and Odc2p[1] are 61% identical in sequence. As described here, they have been overexpressed in *S. cerevisiae* and shown to be isoforms in the inner mitochondrial membrane where they transport C5–C7 oxodicarboxylic acids, including 2-oxoadipate and 2-oxoglutarate, by a counter exchange mechanism. The main physiological roles of these novel transporters are likely to be in cytoplasmic biosynthesis of lysine and glutamate by supplying 2-oxoadipate and 2-oxoglutarate from the mitochondrial matrix and in lysine catabolism.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Deletion of the yeast nuclear genes ODC1 (ORF YPL134c) and ODC2 (ORF Y0R222w) was accomplished by sequential homologous recombination of the auxotrophic markers TRP1 and HIS3 at the respective loci of *S. cerevisiae* YPH499 strain (wild type: MATa ade2–101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801). Deletants were verified by polymerase chain reaction and Western blot analysis. Yeast cells were precultured on synthetic complete medium (8) supplemented with 3% glycerol and 0.1% glucose. Tryptophan, histidine, and uracil were omitted where the genotype permitted. For growth studies, exponentially growing cells were harvested by centrifugation, washed with growth medium and diluted with the same medium until a final optical density of 5×10^{-3} at 600 nm was reached. For the preparation of mitochondria, precultures were diluted 35-fold in YP medium (1% yeast extract, 2% bacto-peptone, pH adjusted to 4.8 with HCl) and grown in the presence of the same carbon sources to mid exponential phase. Galactose (0.45%) was added 6 h before harvesting. For the estimation of the expression of ODCs, yeast cells were grown at 30 °C to mid-log phase in YP medium supplemented with either 2% glucose, 2% galactose, 3% glycerol, 2% ethanol, or 3% lactate and then harvested by centrifugation ($3000 \times g$, 5 min).

Subfractionation of Mitochondria and Quantitative Immunoblotting—Extractions of mitochondria with sodium carbonate or with digitonin were performed as described previously (6). To determine the amount of Odc1p and Odc2p in wild type mitochondria, standard calibration curves were constructed using 10–500 ng of pure recombinant ODC proteins as standards. After transfer of the proteins to the same nitrocellulose membrane, the standards and the mitochondrial samples were immunodecorated simultaneously. Once it had been verified that the sample loading was within the linear range of the calibration curves, the densitometric signal intensity was used to measure the amount of Odc1p and Odc2p.

Construction of the ODC Expression Plasmids—The coding sequences of ODC1 and ODC2 were amplified from *S. cerevisiae* genomic DNA by polymerase chain reaction. Forward and reverse oligonucleotide primers were synthesized corresponding to the extremities of the ODC sequences with additional *Hin*dIII and *Bam*HI sites, respectively. The reverse primers also contained 18 additional bases encoding a six-histidine tag immediately before the translational termination codon. The products of polymerase chain reaction were cloned into the expression vector pYES2 (Invitrogen, Groningen, The Netherlands).

* This work was supported in part by the Consiglio Nazionale delle Richerche Target Project on Biotechnology, by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, by the Piano Biomedicina, Progetto 1 Cluster C04 legge 488/92, and by the European Social Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The names ODC1 and ODC2 (corresponding to ORF YPL134c and ORF YOR222w, respectively) have been reserved for the genes encoding the two isoforms of the yeast oxodicarboxylate carrier.

The resulting expression plasmids (pODC1 or pODC2) were introduced in the odc1Δodc2Δ double mutant, and transformants (odc1Δodc2Δ/pODC1 or odc1Δodc2Δ/pODC2 cells) were selected for uracil auxotrophy. Other experimental conditions have been described before (9).

Overexpression in S. cerevisiae and Purification of the ODC Proteins—Mitochondria were isolated from odc1Δodc2Δ/pODC1 or odc1Δodc2Δ/pODC2 cells according to standard procedures (10) and solubilized in buffer A (500 mM NaCl, 10 mM PIPES, pH 7.0)[2] containing 0.8% digitonin (w/v) and 0.1 mM phenylmethylsulfonyl fluoride at a final concentration of 0.2–0.4 mg protein/ml. After incubation for 20 min at 4 °C, the mixture was centrifuged (138,000 × *g*, 20 min). The supernatant (1.1 ml) was mixed for 1 h at 4 °C with 0.45 ml of nickel-nitrilotriacetic-agarose (Qiagen, Hilden, Germany) previously equilibrated with buffer A. Then the resin was packed into a column (0.5-cm internal diameter) and washed extensively with the following buffers: B, 500 mM NaCl, 0.8% digitonin, 10 mM imidazole, 0.5% Triton X-100, 7.5% glycerol, 10 mM PIPES, pH 7.5 (2 ml); C, 300 mM NaCl, 0.8% digitonin, 10 mM imidazole, 0.1% Triton X-100, 5% glycerol, 10 mM PIPES, pH 7.5 (2 ml); D, 100 mM NaCl, 0.6% digitonin, 10 mM imidazole, 0.05% Triton X-100, 1% glycerol, 10 mM PIPES, pH 7.5 (1 ml); and E, 50 mM NaCl, 0.3% digitonin, 10 mM imidazole, glycerol 0.5%, 10 mM PIPES, pH 7.0 (1 ml). Finally pure ODC proteins were eluted with a buffer containing 50 mM NaCl, 0.1% digitonin, 80 mM imidazole, and 10 mM PIPES, pH 7.0. Protein concentrations were determined by the Lowry method modified for the presence of detergent (11) or by laser densitometry (9).

Protein Chemical Characterization of Overexpressed ODC Isoforms—Proteins were analyzed by SDS-PAGE in 17.5% gels (12) and either stained with Coomassie blue dye or transferred to nitrocellulose membranes. The identities of purified Odc1p and Odc2p were confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry of trypsin digests of the corresponding bands excised from a Coomassie-stained gel. Western blotting was carried out with rabbit antibodies against the bacterially expressed ODC proteins. The overproduction of ODC isoforms as inclusion bodies in the bacterial cytosol and the purification of the inclusion bodies in host strain *Escherichia coli* C0214(DE3) have been described previously (9). The films were scanned with an LKB 2202 Ultroscan laser densitometer.

Reconstitution of the ODC Proteins into Liposomes—Purified ODC proteins were reconstituted by cyclic removal of the detergent with a hydrophobic column (13). The composition of the initial mixture used for reconstitution was: 200 μl of purified isoform (0.3–0.4 μg of protein), 70 μl of 10% Triton X-114, 100 μl of 10% phospholipids in the form of sonicated liposomes, 20 mM oxoglutarate (except where otherwise indicated), 10 mM PIPES, pH 7.0, 0.7 mg of cardiolipin (Sigma), and water to a final volume of 700 μl. These components were mixed throughly, and the mixture was recycled 13 times through an Amberlite column (Supelco) (3.0 × 0.5 cm) pre-equilibrated with a buffer containing 10 mM PIPES, pH 7.0, and with the substrate at the same concentration as in the starting mixture. All operations were performed at 4 °C, except the passages through Amberlite, which were carried out at room temperature.

Transport Measurements—External substrate was removed from proteoliposomes on a Sephadex G-75 column preequilibrated with buffer F (50 mM NaCl and 10 mM PIPES, pH 7.0). Transport at 25 °C was started by adding [^{14}C]oxoglutarate (unless otherwise indicated) to the proteoliposomes and terminated by addition of 30 mM pyridoxal 5′-phosphate and 10 mM bathophenanthroline (the "inhibitor stop" method (13)). In controls, inhibitors were added with the labeled substrate. The external radioactivity was removed on Sephadex G-75, and the internal radioactivity was measured. The transport activity was the difference between experimental and control values. The initial rate of transport was calculated in mmol/min/g protein from the time course of isotope equilibration (13). Various other transport activities were also assayed by the inhibitor stop method. For efflux measurements, the internal substrate pool of the proteoliposomes was made radioactive by carrier-mediated exchange equilibration (13) with 0.1 mM [^{14}C]oxoglutarate added at high specific radioactivity. After 60 min, the residual external radioactivity was removed by passing the proteoliposomes again through a column of Sephadex G-75. Efflux was started by adding unlabeled external substrate or buffer F alone and terminated by adding the inhibitors indicated above.

[2] The abbreviations used are: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PAGE, polyacrylamide gel electrophoresis; ODC, oxodicarboxylate carrier.

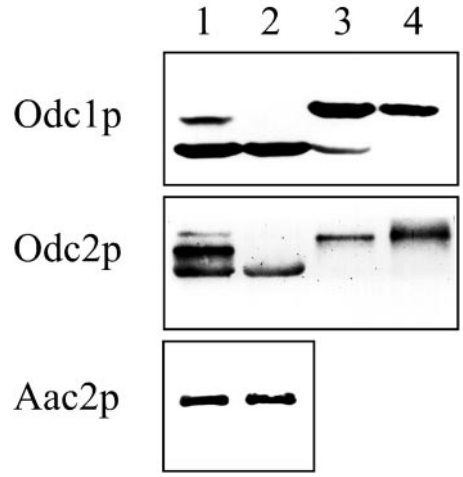

FIG. 1. **Immunoblot analysis of ODC proteins in yeast mitochondria.** 25 μg of mitochondrial protein from wild type (*lane 1*) and odc1Δ odc2Δ cells (*lane 2*) were separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antibodies directed against Odc1p, Odc2p and the ADP/ATP carrier (Aac2p). *Lane 3*, 10 and 1 μg of mitochondrial protein from odc1Δodc2Δ/pODC1 and odc1Δodc2Δ/pODC2 cells, respectively. *Lane 4*, 350 ng (Odc1p) and 5 ng (Odc2p) of recombinant ODC transport proteins purified from mitochondria in *lane 3*.

RESULTS

Subcellular Localization of the ODC Proteins—Immunoreactive bands on SDS-PAGE gels were detected with antibodies against Odc1p and Odc2p. Bands with apparent molecular masses of about 36.5 and 35.0 kDa, respectively, were detected in wild type mitochondria (Fig. 1, *lane 1*) but not in mitochondria from the odc1Δodc2Δ double mutant (Fig. 1, *lane 2*). The antibody against Odc2p cross-reacted with Odc1p (upper band), and both antibodies reacted with an unidentified band of about 33.0 kDa that was also present in the odc1Δodc2Δ mitochondria. The contents of the ADP/ATP carrier and (not shown) the phosphate, succinate-fumarate and dicarboxylate carriers detected with specific antibodies were essentially the same in both wild type and odc1Δodc2Δ mitochondria. Therefore, the absence of both ODC proteins from the double mutant does not affect the expression of other mitochondrial carriers. Furthermore, the phenotype of the odc1Δodc2Δ strain was studied by comparison of the growth of the mutant cell with the parental strain in shake flask cultures on different media. Both the wild type and the deletion strain yeast exhibited substantial and similar growth on either rich medium (YP) or synthetic complete medium supplemented with either 2% glucose, 2% galactose, 3% glycerol, 2% ethanol or 3% lactate, indicating that the absence of the ODC proteins does not impair the respiratory function of mitochondria.

The submitochondrial location of Odc1p and Odc2p was examined by separation of soluble and peripheral proteins from integral membrane proteins of wild type mitochondria by carbonate treatment (Fig. 2). Both Odc1p and Odc2p remained in the membrane protein fraction, as did the ADP/ATP carrier and (not shown) Tom40p (marker proteins of inner and outer mitochondrial membranes, respectively), but the matrix protein hsp70 and (not shown) the intermembrane space protein cytochrome b_2 were in the soluble and peripheral protein fraction. In other experiments, at 0.3% digitonin more than 80% of the outer membrane protein Tom40p, and less than 10% of Odc1p, Odc2p and the ADP/ATP carrier, were solubilized from wild type mitochondria. At higher levels of digitonin, Odc1p and Odc2p were solubilized progressively in parallel with the ADP/ATP carrier (data not shown). Therefore, Odc1p and

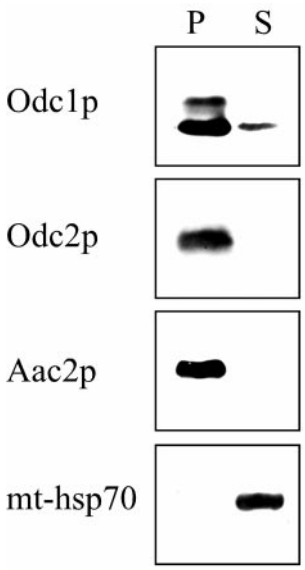

FIG. 2. **Submitochondrial localization of Odc1p and Odc2p.** Analysis by SDS-PAGE and Western blotting of soluble and peripheral proteins (*S*) and intrinsic membrane proteins (*P*) from yeast mitochondria (5 μg of protein in each slot) blotted with antisera directed against Odc1p, Odc2p, the ADP/ATP carrier (Aac2p; inner membrane component), and the mitochondrial hsp70 (mt-hsp70; matrix protein). In the *top two panels* the reaction was terminated once Odc1p and Odc2p became visible.

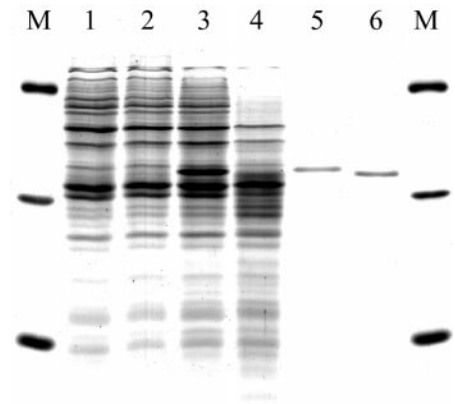

FIG. 3. **Purification of the overexpressed ODC proteins.** Proteins were separated by SDS-PAGE and stained with Coomassie blue dye. *Lanes M*, markers (bovine serum albumin, carbonic anhydrase and cytochrome *c*). *Lanes 1–4*, mitochondrial protein (100 μg) from wild type (*lane 1*), odc1Δodc2Δ mutant (*lane 2*), odc1Δodc2Δ/pODC1 (*lane 3*), and odc1Δodc2Δ/pODC2 (*lane 4*) strains. Cells were harvested 6 h after addition of galactose. *Lanes 5* and *6*, 2 μg of Odc1p (*lane 5*) and 4 μg of Odc2p (*lane 6*) purified from mitochondria in *lanes 3* and *4*, respectively.

TABLE I

Oxoglutarate homoexchange in liposomes reconstituted with mitochondrial extracts from various yeast strains

Proteoliposomes were preloaded internally with 20 mM oxoglutarate. Transport was started by the external addition of 0.1 mM [^{14}C]oxoglutarate. The data represent the means ± S.D. of at least three different experiments.

Strain	[^{14}C]Oxoglutarate uptake
	μmol/min/g protein
Wild type	7.2 ± 2.0
odc1Δodc2Δ	0.8 ± 0.2
odc1Δodc2Δ/pODC1	85.0 ± 14.9
odc1Δodc2Δ/pODC2	26.8 ± 5.6
odc1Δodc2Δ/pOAC1	1.4 ± 0.4
odc1Δodc2Δ/pCRC1	1.0 ± 0.3

Odc2p are integral proteins of the inner mitochondrial membrane.

Expression in S. cerevisiae and Purification of the ODC Proteins—Odc1p and Odc2p were overexpressed at high levels in a *S. cerevisiae* strain devoid of both corresponding genes (odc1Δodc2Δ strain) (Fig. 3, *lanes 3* and *4*). Their apparent molecular masses were about 38 and 37 kDa (the calculated values including the initiator methionine and the histidine tail were 35,006 and 34,807 Da, respectively). The successful overexpression and targeting of the episomal Odc1p and Odc2p to mitochondria was confirmed by Western blotting of isolated mitochondria from the odc1Δodc2Δ/p0DC strains (Fig. 1, *lane 3*), because the amount of mitochondrial protein applied in *lane 3* of Fig. 1 was 2.5 (from odc1Δodc2Δ/p0DC1 cells) and 25 (from odc1Δodc2Δ/p0DC2 cells) times less than the amount of wild type mitochondrial protein applied in *lane 1* of Fig. 1. The differences in the molecular mass of immunodecorated bands in *lane 3* (odc1Δodc2Δ/p0DC mitochondria) and *lane 1* (wild type mitochondria) are a reflection of the presence of a C-terminal histidine tag in the recombinant proteins.

The presence of the histidine tail at the C-terminal end of the expressed ODC isoforms allowed their purification by a Ni^{+}-agarose affinity column (Fig. 3, *lanes 5* and *6*, and Fig. 1, *lane 4*). About 0.7 mg of Odc1p and about 0.1 mg of Odc2p were obtained per liter of culture. The identity of purified Odc1p and Odc2p was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Functional Characterization of Recombinant Odc1p and Odc2p—Proteoliposomes reconstituted with digitonin-solubilized mitochondria isolated from odc1Δodc2Δ/p0DC1 or odc1Δodc2Δ/p0DC2 strains were able to catalyze an active [^{14}C]oxoglutarate/oxoglutarate homoexchange (Table I). A lower oxoglutarate transport was observed upon reconstitution of the digitonin extract from wild type mitochondria, whereas liposomes reconstituted with the extract from odc1Δodc2Δ mitochondria showed a very low but reproducible oxoglutarate exchange. Furthermore, oxoglutarate transport measured upon reconstitution of the mitochondrial extract isolated from the double deletion strain transformed with the pYES2 vector harboring the sequence encoding the yeast oxaloacetate carrier (odc1Δodc2Δ/pOAC1 strain) (6), the yeast carnitine carrier (odc1Δodc2Δ/pCRC1 strain) (7), or with the empty pYES2 vector (not shown) was not significantly increased.

The purified and reconstituted Odc1p and Odc2p catalyzed a very active [^{14}C]oxoglutarate/oxoglutarate exchange, which was inhibited by a mixture of pyridoxal 5′-phosphate and bathophenanthroline. No such activity was found with Odc1p and Odc2p that had been boiled before incorporation into liposomes. Likewise, no [^{14}C]oxoglutarate uptake was observed into proteoliposomes that did not contain internal oxoglutarate, indicating that Odc1p and Odc2p do not catalyze a unidirectional transport (uniport) of oxoglutarate but only the exchange reaction. To obtain further information about the mechanism of transport catalyzed by Odc1p and Odc2p, the efflux of [^{14}C]oxoglutarate from prelabeled active proteoliposomes was investigated because it provides a more convenient assay for unidirectional transport (13). An experiment performed with proteoliposomes reconstituted with Odc1p is shown in Fig. 4. In the absence of external substrate, no efflux was observed even after incubation for 1 h. However, upon addition of external oxoglutarate, an extensive efflux of intraliposomal radioactivity occurred, and this efflux was prevented completely by the presence of the inhibitors pyridoxal 5′-phosphate and bathophenanthroline (Fig. 4). Similar data were obtained using Odc2p instead of Odc1p. These results show clearly that reconstituted Odc1p and Odc2p catalyze an obligatory exchange reaction of internal oxoglutarate for external

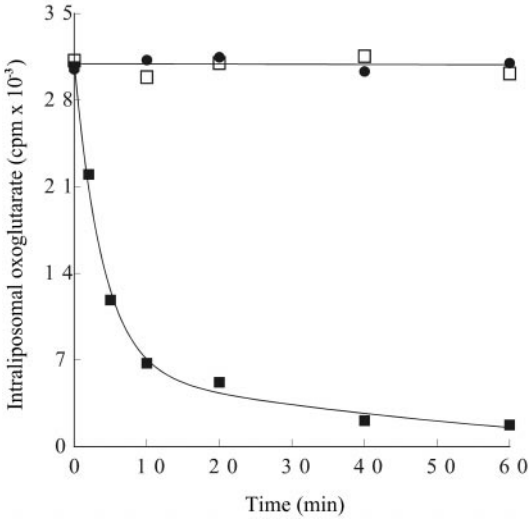

FIG. 4. **Efflux of [^{14}C]oxoglutarate from proteoliposomes.** Proteoliposomes were reconstituted with recombinant Odc1p in the presence of 20 mM oxoglutarate, and then the internal substrate pool was labeled by carrier-mediated exchange equilibration. After removal of external substrate by Sephadex G-75 chromatography, the efflux of [^{14}C]oxoglutarate was started by adding buffer F alone (●) or 10 mM oxoglutarate (■), or 10 mM oxoglutarate, 30 mM pyridoxal 5′-phosphate, and 10 mM bathophenanthroline (□) in the same buffer.

oxoglutarate. Furthermore, the proteoliposomes did not catalyze homoexchanges for glutamate, aspartate, 2-oxoisocaproate, glutamine, ornithine, ADP, phosphate, sulfate, and carnitine (internal concentration, 10 mM; external concentration, 1 mM).

The substrate specificity of purified Odc1p and Odc2p was investigated further by measuring the uptake of [^{14}C]oxoglutarate into proteoliposomes that had been preloaded with various substrates. As shown in Table II, [^{14}C]oxoglutarate was taken up efficiently by proteoliposomes containing internal oxoglutarate, 2-oxoadipate, 2-oxopimelate, glutarate, adipate, pimelate, L-malate, and D-malate. A much lower activity was observed in the presence of internal oxaloacetate, succinate, citrate, and isocitrate. A very low activity was found with internal malonate, suberate, fumarate, and maleate, and (not shown) no exchange was found with oxalate, aspartate, glutamate, 2-aminoadipate, 2-aminopimelate, pyruvate, 2-oxobutyrate, 2-hydroxybutyrate, 2-oxovalerate, 2-oxoisocaproate, phosphate, sulfate, thiosulfate, ADP, ATP, ornithine, glutamine, and carnitine.

The [^{14}C]oxoglutarate/oxoglutarate exchange reactions catalyzed by reconstituted Odc1p and Odc2p were inhibited strongly by mercurials (mersalyl, *p*-chloromercuribenzene sulfonate, and mercuric chloride), by pyridoxal 5′-phosphate, by bathophenanthroline, and by α-cyanocinnamate (Table III). Also, both Odc1p and Odc2p were inhibited considerably by *N*-ethylmaleimide. The impermeable dicarboxylate analogues butylmalonate and phenylsuccinate, which are known to be powerful inhibitors of the oxoglutarate and dicarboxylate carriers (14, 15), decreased the reconstituted transport activities rather poorly. Also, the tricarboxylate analogue 1,2,3-benzenetricarboxylate, a very efficient inhibitor of the citrate carrier (16), had a rather mild inhibitory effect, and carboxyatractyloside, a powerful inhibitor of the ADP/ATP carrier (17), had little or no effect on the activities of Odc1p and Odc2p.

In addition, the ability of nonradioactive potential substrates to inhibit the oxoglutarate/oxoglutarate exchange was examined. The effectiveness of dicarboxylates and 2-oxodicarboxylates with different carbon chain length on the rate of oxoglutarate uptake are compared in Fig. 5. With both reconstituted

TABLE II

Dependence on internal substrate of the transport properties of proteoliposomes reconstituted with recombinant Odc1p or Odc2p

Proteoliposomes were preloaded internally with various substrates (concentration, 20 mM). Transport was started by adding [^{14}C]oxoglutarate (final concentration, 0.6 mM) and terminated after 45 s. Similar results were obtained in at least three independent experiments.

Internal substrate	[^{14}C]Oxoglutarate transport	
	Odc1p	Odc2p
	mmol/min/g protein	
None (Cl^- present)	0.4	0.2
Oxaloacetate	31.8	5.5
2-Oxoglutarate	112.5	27.8
2-Oxoadipate	85.0	22.1
2-Oxopimelate	71.6	14.8
Malonate	4.8	2.2
Succinate	19.1	4.3
Glutarate	81.1	17.6
Adipate	65.0	16.7
Pimelate	48.4	10.4
Suberate	2.8	0.4
L-Malate	56.4	11.4
D-Malate	52.1	10.5
Fumarate	8.9	2.4
Maleate	7.1	2.6
Citrate	28.6	8.0
L-Isocitrate	19.4	3.7

TABLE III

Effect of inhibitors on the [^{14}C]oxoglutarate/oxoglutarate exchange by proteoliposomes reconstituted with Odc1p and Odc2p

Proteoliposomes were preloaded internally with 20 mM oxoglutarate, and transport was initiated by the addition of 0.4 mM [^{14}C]oxoglutarate. The incubation time was 45 s. Thiol reagents and α-cyanocinnamate were added 2 min before the labeled substrate; the other inhibitors and external substrates were added together with [^{14}C]oxoglutarate. The final concentration of the inhibitors was 4 mM, except for mercurials (10 μM), carboxyatractyloside (0.1 mM), and *N*-ethylmaleimide and α-cyanocinnamate (2 mM). Similar results were obtained in three independent experiments in duplicate.

Reagents	Inhibition	
	Odc1p	Odc2p
	%	
Mersalyl	99	100
p-Chloromercuriphenylsulfonate	98	100
$HgCl_2$	100	98
N-Ethylmaleimide	65	43
Pyridoxal 5′-phosphate	87	92
Bathophenanthroline	97	89
Butylmalonate	18	25
Phenylsuccinate	23	22
1,2,3-Benzenetricarboxylate	41	37
α-Cyanocinnamate	86	80
Carboxyatractyloside	7	10

Odc1p and (not shown) Odc2p, glutarate, adipate, and pimelate with 5–7 carbon atoms caused a significant inhibition of oxoglutarate uptake, whereas oxalate, malonate, succinate, and suberate had virtually no effect. The presence of a carbonyl group on the dicarboxylate molecule enhanced the inhibitory effect of the compounds with a maximum of inhibition at six carbon atoms. In similar experiments (not shown) the effect of other dicarboxylates on the rate of oxoglutarate uptake catalyzed by the recombinant and reconstituted Odc1p and Odc2p was also tested. The presence of a hydroxyl group on the C_4 dicarboxylate molecule (as in malate) increased about 10-fold the extent of the inhibition of oxoglutarate uptake with respect to the corresponding dicarboxylate. Also, L- and D-tartrate inhibited the uptake of oxoglutarate more efficiently than succinate, their inhibitory effect being comparable with that of malate. However, the C_5 hydroxydicarboxylate, hydroxyglutarate, was slightly less effective than glutarate, and the C_3 hydroxydicarboxylate (tartronate) was completely ineffective

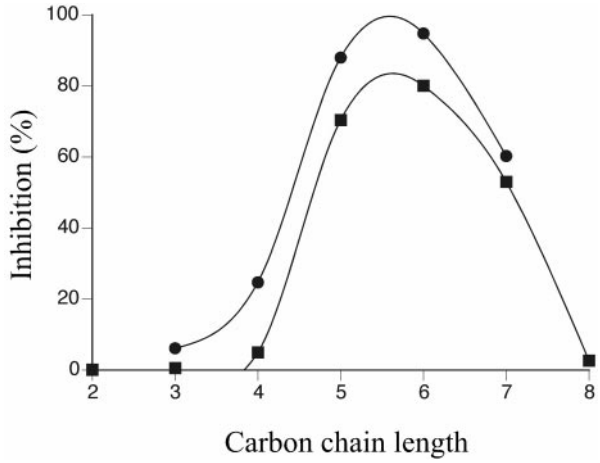

FIG. 5. **Effect of dicarboxylates and 2-oxodicarboxylates with different carbon chain length on the rate of [^{14}C]oxoglutarate uptake into proteoliposomes reconstituted with recombinant Odc1p.** Proteoliposomes were preloaded with 20 mM oxoglutarate and reconstituted with recombinant Odc1p. Transport was initiated by the addition of 0.4 mM [^{14}C]oxoglutarate and terminated after 45 s. Oxalate, malonate, succinate, glutarate, adipate, pimelate, and suberate (■) and the corresponding 2-oxoacids (●) were added simultaneously with [^{14}C]oxoglutarate at 4.0 mM concentration. The control value for uninhibited 2-oxoglutarate uptake was 108 mmol/min/g protein. The results are given as a percentage of inhibition of the control. Similar results were obtained in three separate experiments in duplicate.

like the corresponding dicarboxylate. Both the *cis*- and *trans*-unsaturated dicarboxylates, fumarate and maleate, had no effect, nor had the aminodicarboxylates. D,L-Threo-hydroxyaspartate has been reported to inhibit the uptake of oxoglutarate into intact yeast mitochondria (18), but it had no effect on oxoglutarate transport catalyzed by reconstituted Odc1p and Odc2p. In view of the inhibition of oxoglutarate uptake by dicarboxylates carrying a carbonyl group, the effect of oxomonocarboxylates on the rate of uptake of 0.4 mM oxoglutarate was also tested. Pyruvate, 2-oxovalerate 2-oxobutyrate, 2-oxoisocaproate, and 5-oxohexanoate (all at 8 mM concentration, *i.e.* 20 times greater than the substrate) did not influence the rate of oxoglutarate uptake (data not shown). Finally, several aminomonocarboxylates, (glycine, alanine, valine, threonine, and serine), and other amino acids (glutamine, asparagine, lysine, arginine, histidine, and ornithine) had no effect on the oxoglutarate/oxoglutarate exchange (data not shown).

Kinetic Characteristics of Recombinant Odc1p and Odc2p—The kinetic constants of the recombinant purified Odc1p and Odc2p were determined by measuring the initial transport rate at various external [^{14}C]oxoglutarate concentrations, in the presence of a constant saturating internal concentration of 20 mM oxoglutarate. The transport affinities (K_m) and the specific activities (V_{max}) for the oxoglutarate/oxoglutarate exchange at 25 °C, were 0.52 ± 0.08 mM and 252 ± 53 mmol/min/g protein for Odc1p (24 experiments) and 0.47 ± 0.07 mM and 73 ± 17 mmol/min/g protein for Odc2p (16 experiments). All of the compounds summarized in Table IV inhibited oxoglutarate uptake by both isoforms competitively, because they were found to increase the apparent K_m without changing the V_{max} of oxoglutarate uptake (not shown). The inhibition constants (K_i) of 2-oxoadipate are only about 2-fold lower than the K_m of oxoglutarate but are about 5–7 times lower than the K_i values of malate and 2-oxopimelate and more than 50 times lower than those of oxaloacetate and succinate. The K_i values of malate and citrate are similar to the K_m values of the same substrates for the reconstituted Odc1p, as determined from Lineweaver-Burk plots of the rate of [^{14}C]malate or [^{14}C]citrate uptake in the presence of a constant internal oxoglutarate

TABLE IV
Competition with [^{14}C]oxoglutarate uptake in proteoliposomes containing recombinant yeast Odc1p and Odc2p

The values were calculated from Lineweaver-Burk plots of the rate of [^{14}C]oxoglutarate *versus* substrate concentrations. The competing substrates at appropriate constant concentrations were added together with 0.1–2.0 mM [^{14}C]oxoglutarate to proteoliposomes containing 20 mM oxoglutarate and reconstituted with recombinant Odc1p or Odc2p. The data represent the means ± S.D. of at least three different experiments. ND, not determined.

Substrate	K_i	
	Odc1p	Odc2p
	mM	
2-Oxoadipate	0.26 ± 0.03	0.31 ± 0.04
2-Oxopimelate	1.83 ± 0.20	1.64 ± 0.18
L-Malate	1.32 ± 0.13	1.37 ± 0.16
Citrate	6.0 ± 0.8	5.9 ± 0.6
Oxaloacetate	>15	>15
Succinate	>15	ND
Isocitrate	>15	ND

concentration of 20 mM. Under these conditions the K_m of malate was 1.3 ± 0.2 mM (seven experiments), and that of citrate was 5.7 ± 0.5 mM (three experiments). Taken together these results demonstrate that 2-oxoadipate and 2-oxoglutarate are the best substrates for reconstituted Odc1p and Odc2p.

Influence of the Carbon Source on the Expression of ODC Proteins—Because Odc1p and Odc2p appear to have virtually the same transport properties, to shed light on the metabolic significance of the ODC isoforms the regulation of protein expression was examined. To quantify Odc1p and Odc2p, various amounts of mitochondrial samples from yeast cells fed on glycerol were loaded onto the gel and immunoblotted simultaneously with the appropriate range of bacterially expressed Odc1p and Odc2p standards (see "Experimental Procedures"). In four determinations, the abundance of ODC proteins was 123 ± 30 pmol/mg of protein of Odc1p and 9 ± 2 pmol/mg of protein of Odc2p.

The expression of Odc1p and Odc2p was investigated by immunoblot analysis of mitochondria isolated from the wild type strain following growth on different carbon sources. The expression of Odc1p is repressed strongly by glucose, whereas Odc2p appears to be expressed at comparatively higher levels on glucose and galactose medium than on medium supplemented with nonfermentable carbon sources (Fig. 6).

DISCUSSION

Kinetic Properties of ODC Isoforms—The transport characteristics and kinetic parameters of the ODC proteins show that they are isoforms of a novel mitochondrial transporter for C5–C7 oxodicarboxylates with greatest specificity for 2-oxoadipate and 2-oxoglutarate. ODC also transports the corresponding dicarboxylates and to a lesser extent malate and citrate.

The substrate specificity of the yeast ODC isoforms is distinct from that of any other previously characterized mitochondrial carrier. It differs from that of the succinate-fumarate carrier (19), which is its closest sequence homologue (5), because the former transports fumarate and succinate with a very low efficiency (K_m > 15 mM). ODC is also quite different from the mammalian oxoglutarate carrier. First, the yeast ODC isoforms and the bovine oxoglutarate carrier have sequence identities of 24 and 25%, indicating that they are not orthologues. Second, the ODCs transport C5–C7 oxodicarboxylates, whereas the mammalian oxoglutarate carrier transports C4 and C5 oxodicarboxylates (14, 20, 21). Third, the ODC works best with C5–C7 dicarboxylates, whereas the mammalian oxoglutarate carrier displays optimal transport activity with C3 and C4 dicarboxylates (malonate, succinate, and ma-

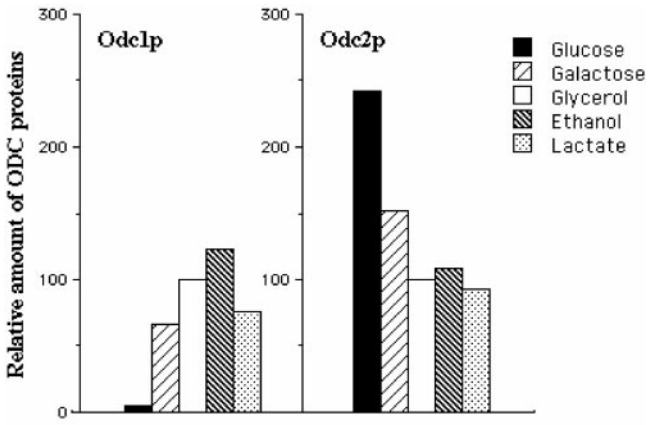

FIG. 6. **Comparison of the expression of Odc1p and Odc2p on various carbon sources.** Cells were harvested from exponentially growing cells on YP medium supplemented with the indicated carbon sources. Amounts of ODC proteins were estimated by densitometry upon immunodecoration of mitochondrial proteins with specific antisera. Similar results were obtained in three independent experiments in duplicate. The amount of Odc1p and Odc2p present in mitochondria from glycerol-fed cells was taken as 100%.

leate) (14, 20, 21). Fourth, ODC appears to be less stereospecific than the mammalian oxoglutarate carrier as it has equal affinity for L- and D-malate, whereas the mammalian carrier has little or no affinity for the D-stereoisomer (14, 20, 21). Fifth, both isoforms of ODC accept the tricarboxylates citrate and isocitrate as substrates, although with low affinity, whereas the specificity of the mammalian oxoglutarate carrier is confined to dicarboxylates (14, 20, 21).

Regulation of Expression of the ODC Proteins—Many yeast genes involved in the tricarboxylic acid cycle, in oxidative phosphorylation and in ATP generation are subject to repression by fermentable carbon sources (catabolite repression). Under nonrepressed conditions in cells fed on glycerol, Odc1p is about 15-fold more abundant than Odc2p. However, ODC1 expression is strongly repressed by catabolites, whereas ODC2 appears to be expressed at a higher level in the presence of galactose and glucose (Fig. 6). This apparent increase could result from the repression of most other mitochondrial proteins rather than from induction of the ODC2 itself. An increase in ODC1 expression has been observed without significant change in ODC2 expression during the diauxic shift when a culture of *S. cerevisiae* growing on glucose in batch culture exhausted the glucose supply and began to oxidize ethanol produced by fermentation (22). A temporal pattern of expression similar to that of ODC1 was observed for AAC1 and AAC2, the gene for the major "aerobic" isoform of ADP/ATP translocase, whereas the transcript level of AAC3, which is induced under anaerobic conditions, remained constant (22). These considerations indicate that Odc1p is the major ODC isoform under respiratory conditions and that Odc2p is the prevailing isoform in the presence of glucose and possibly in anaerobiosis. It should be noted that ODC1, AAC2 (encoding an isoform of ADP/ATP translocase), and MIR1 (encoding the phosphate carrier) are the only yeast mitochondrial carrier genes that increase their expression following adaptive evolution in aerobic glucose-limited conditions (23), indicating that they all have key functions in cell metabolism.

Role of ODC in Lysine Metabolism—In yeast, lysine is synthesized via the α-aminoadipate pathway, whereby 2-oxoadipate is produced in the mitochondrial matrix and 2-aminoadipate is converted into lysine in the cytoplasm (24). The results reported here suggest that 2-oxoadipate is exported by ODC from the mitochondrial matrix to the cytoplasm where it is transaminated to 2-aminoadipate (Fig. 7). Because ODC functions by a strict exchange mechanism, the carrier-mediated efflux of 2-oxoadipate requires uptake of a counter-substrate. On the basis of transport measurements (Table II), 2-oxoglutarate, malate, or another transported Krebs cycle intermediate (according to the metabolic conditions) can fulfill this role and satisfy an important anaplerotic role by compensating the Krebs cycle for the 2-oxoadipateglutarate withdrawn for 2-oxoadipate synthesis.

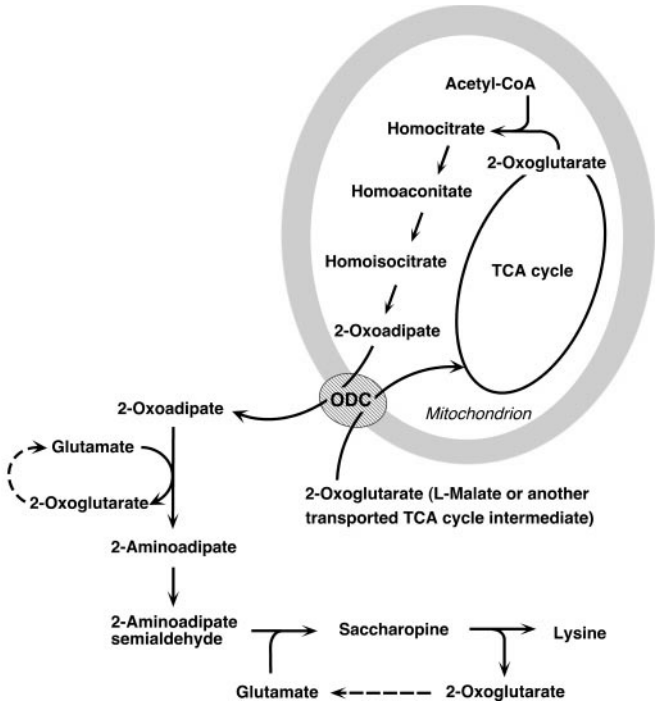

FIG. 7. **Compartmentalization of selected enzymes involved in lysine biosynthesis in *S. cerevisiae* and the role of the mitochondrial oxodicarboxylate carrier.** The *dashed lines* indicate the entry of nitrogen into the glutamate molecule.

Reversal of the cytoplasmic part of the 2-aminoadipate pathway is used in lysine catabolism in both *S. cerevisiae* and animals (24, 25). For this purpose, it is likely that 2-oxoadipate is imported by ODC into mitochondria first to be converted into glutaryl-CoA by 2-oxoadipate dehydrogenase (a mitochondrial enzyme) and then metabolized in a series of steps to acetyl-CoA. In mammals, cytosolic 2-oxoadipate is also produced by catabolism of tryptophan (26) and possibly hydroxylysine. Therefore, it is likely that an ODC protein exists in man and that defects in its activity could be linked to 2-ketoadipic acidemia. It has been suggested that this inborn error of catabolism of lysine, tryptophan, and hydroxylysine (27) may be due to 2-oxoadipate dehydrogenase deficiency, but such a defect has not been demonstrated directly.

The yeast strains used in the present investigation are devoid of the LYS2 gene, encoding the major subunit of aminoadipate reductase (α-aminoadipate-semialdehyde dehydrogenase), and so confirmation of the proposed involvement of the ODC proteins in lysine metabolism requires construction of appropriate strains with a functional LYS2.

Role of ODC in Nitrogen Assimilation—In *S. cerevisiae,* nitrogen in the form of ammonium is assimilated either by the action of isoforms 1 and 3 of glutamate dehydrogenase or by the action of glutamine synthetase and glutamate synthase together (28). These enzymes are cytoplasmic (29, 30), and nitrogen assimilation requires the carbon skeleton of oxoglutarate. The major site of oxoglutarate production is the mitochondrial matrix, and therefore, another physiological role of ODC isoforms is probably to export it to the cytoplasm. It should be

stressed that it is unlikely that *S. cerevisiae* has an orthologue to the mammalian 2-oxoglutarate carrier (6). The only yeast carriers to cluster on a phylogenetic tree with the mammalian oxoglutarate carrier (5) have been identified as the dicarboxylate and oxaloacetate-sulfate carriers (4, 6). Because the odc1Δodc2Δ strain grew on different fermentable and nonfermentable carbon sources at rates similar to the parental strain, the mitochondrial ODC proteins are not indispensable for respiration, but this does not imply that the ODC is not involved in cytosolic glutamate formation. The synthetic media used in this study contained glutamate, which may suffice to sustain the growth of mutant cells. Thus, more stringent growth conditions may be required to observe a phenotype. Also, yeast has alternative mechanisms for generating cytosolic 2-oxoglutarate to support nitrogen assimilation. It is possible that impairment of oxoglutarate export from mitochondria may be circumvented by the cytosolic NADP-dependent isocitrate dehydrogenase, Idp2p, which is sufficient for growth of *S. cerevisiae* without glutamate in the absence of the mitochondrial isozymes when oxoglutarate cannot be generated in the matrix (31). Another possibility is that yeast mitochondria contain a second unidentified oxoglutarate transporter.

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Identification in *Saccharomyces cerevisiae* of Two Isoforms of a Novel Mitochondrial Transporter for 2-Oxoadipate and 2-Oxoglutarate

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J. Biol. Chem. 2001, 276:1916-1922.
doi: 10.1074/jbc.M004332200 originally published online September 29, 2000
