THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 32, Issue of August 6, pp. 22184–22190, 1999

Printed in U.S.A.

Identification of the Yeast Mitochondrial Transporter for Oxaloacetate and Sulfate*

(Received for publication, April 12, 1999, and in revised form, May 14, 1999)

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***Saccharomyces cerevisiae* encodes 35 members of the mitochondrial carrier family, including the OAC protein. The transport specificities of some family members are known, but most are not. The function of the OAC has been revealed by overproduction in *Escherichia coli*, reconstitution into liposomes, and demonstration that the proteoliposomes transport malonate, oxaloacetate, sulfate, and thiosulfate. Reconstituted OAC catalyzes both unidirectional transport and exchange of substrates. In *S. cerevisiae*, OAC is in inner mitochondrial membranes, and deletion of its gene greatly reduces transport of oxaloacetate sulfate, thiosulfate, and malonate. Mitochondria from wild-type cells swelled in isoosmotic solutions of ammonium salts of oxaloacetate, sulfate, thiosulfate, and malonate, indicating that these anions are cotransported with protons. Overexpression of OAC in the deletion strain increased greatly the [^{35}S]sulfate/sulfate and [^{35}S]sulfate/oxaloacetate exchanges in proteoliposomes reconstituted with digitonin extracts of mitochondria. The main physiological role of OAC appears to be to use the proton-motive force to take up into mitochondria oxaloacetate produced from pyruvate by cytoplasmic pyruvate carboxylase.**

Saccharomyces cerevisiae has two principal pathways for replenishing the intermediates of the Krebs cycle that have been withdrawn for biosynthesis. They are the glyoxylate cycle and the carboxylation of pyruvate to oxaloacetate, catalyzed by two cytosolic isozymes of pyruvate carboxylase. (Mammalian pyruvate carboxylase is in the mitochondrial matrix.) These processes require traffic of substrates across the inner mitochondrial membrane. *S. cerevisiae* encodes 35 members of the mitochondrial carrier family (1–4), including the dicarboxylate and succinate-fumarate carriers (5, 6). The former catalyzes the import into mitochondria of succinate (or malate) in exchange for phosphate, producing a net uptake of succinate and supply of a substrate to the Krebs cycle (7). The latter exchanges external succinate for fumarate and is required for gluconeogenesis (6, 8). Yeast gene disruption strains (Δ-*DIC1* and Δ-*SFC1*) cannot grow on either ethanol or acetate as sole carbon source, but they grow well on glycerol, pyruvate and other nonfermentable substrates (7, 9). Growth of the Δ-*DIC1* strain on ethanol or acetate is restored by both low concentrations of oxaloacetate and other compounds that start the tricarboxylate cycle (and the oxidation of the acetyl-CoA unconsumed by the glyoxylate cycle) by generating either intramitochondrial oxaloacetate or other Krebs cycle intermediates (7). Therefore, does oxaloacetate enter yeast mitochondria, and if so, how?

The *S. cerevisiae* gene *OAC1*[1] (formerly known as *PMT* or *YKL120w*) encodes a member of the mitochondrial carrier family of hitherto unknown function that is 29% identical to the yeast DIC, its closest relative in yeast and 30% identical to the bovine 2-oxoglutarate/malate carrier. OAC and the dicarboxylate carrier are the only carriers that cluster on a phylogenetic tree with the bovine oxoglutarate/malate carrier (10, 11). Disruption of the *OAC1* gene produced no phenotype on rich glycerol medium (12), and its transcript level was higher in synthetic than in rich medium (13).

We have overexpressed the OAC in *E. coli*, reconstituted it into phospholipid vesicles, and shown that it transports oxaloacetate, sulfate, and thiosulfate both *in vitro* and *in vivo*. One of its main functions is probably to carry oxaloacetate produced by cytoplasmic pyruvate carboxylase into the mitochondrial matrix.

EXPERIMENTAL PROCEDURES

Materials—[2-^{14}C]Malonic acid, α-[1-^{14}C]ketoglutaric acid, [1,5-[^{14}C] citric acid, L-[^{14}C(U)]glutamic acid, L-[^{14}C(U)]aspartic acid, L-[^{14}C (U)] glutamine and [8-^{14}C]adenosine 5′-diphosphate trisodium salt were supplied by NEN Life Science Products. L-[1,4(2, 3)-^{14}C]Malic acid, [^{35}S]sulfate, [^{32}P]phosphate, and L-[*methyl*-^{3}H]carnitine were obtained from Amersham Pharmacia Biotech, and [2,3-^{14}C]fumaric acid and L-[2,3-^{3}H]ornithine were from Sigma. Egg yolk phospholipids were purchased from Fluka, and cardiolipin and *N*-lauroylsarcosine were from Sigma. Amberlite XAD-2 was supplied by Supelco (Milan, Italy). The vector pYeDP60 was a generous gift of Dr. D. Pompon (Gif-sur-Yvette, France).

Amplification of the OAC1 Gene from Yeast Genomic DNA—Oligonucleotide primers were synthesized with sequences corresponding to the extremities of the coding sequence for OAC (nucleotides 216,990–217,964 on the positive strand of chromosome XI), with additional *Nde*I and *Hin*dIII sites. Other conditions have been given before (14).

Bacterial Expression of OAC—The overproduction of OAC as inclusion bodies in the bacterial cytosol and the purification of the inclusion bodies (5, 15) in host strain *E. coli* C0214(DE3) (14), the analysis of proteins by SDS-PAGE[2] in 17.5% gels (16), and N-terminal sequencing (14) have been described previously. The yield of purified yeast protein/liter of bacterial culture was estimated by laser densitometry (14).

* The work at the University of Bari was supported by the Consiglio Nazionale delle Ricerche Target Project on "Biotechnology," by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ238698.

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[1] The name *OAC1* has been reserved for the gene encoding the yeast oxaloacetate carrier.

[2] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

Reconstitution of the Recombinant OAC into Liposomes and Measurement of Its Transport Activity—The OAC was reconstituted into proteoliposomes as described for homologues (14, 17). External substrate was removed from proteoliposomes on Sephadex G-75. Transport at 25 °C was started by adding [^{14}C]malonate or [^{35}S]sulfate to the proteoliposomes and terminated by addition of 0.1 mM *p*-chloromercuribenzene sulfonate and 10 mM bathophenanthroline (the "inhibitor-stop" method (18)). In controls, inhibitors were added with the labeled substrate. Finally, the external radioactivity was removed on Dowex AGl-X8, and the internal radioactivity was measured (18). The transport activity was the difference between experimental and control values. The initial rate of transport was calculated in μmol/min/g of protein from the time course of isotope equilibration (18). Various other transport activities were also assayed by inhibitor-stop.

Yeast Strains and Media—The *OAC1* gene was deleted by homologous recombination of the auxotrophic marker URA3 at the *OAC1* gene locus of *S. cerevisiae* strain YPH499. Its genotype is MATa *ade*2–101 *his*3-Δ 200 *leu*2-Δ1 *ura*3–52 *trp*l-Δ 63 *lys2*-801 OAC1::URA3. The coding sequence was cloned into the yeast expression plasmid pYeDP60 (19) yielding plasmid *OAC1*-pYeDP60. It was introduced into the Δ-*OAC1* yeast strain, and transformants were selected for adenine auxotrophy. Wild-type fungi and the deleted strain were grown either in rich medium containing 2% bactopeptone and 1% yeast extract (YP) or in synthetic complete medium (20) supplemented with either fermentatable or nonfermentable carbon sources (2% glucose or 2% galactose, and 3% glycerol, 2% ethanol, and 3% acetate or 2% lactate). The Δ-*OAC1* strain transformed with the *OAC1*-pYeDP60 construct (the *OAC1*-pYeDP60 strain) was precultured in synthetic complete medium lacking uracil, supplemented with 2% ethanol. For preparation of mitochondria, the preculture was diluted 35-fold in YP medium. The *Gal-Cyc* promoter was repressed by addition of 0.1% glucose. The *OAC1*-pYeDP60 cells were grown to exponential phase, and galactose (0.4%) was added 6 h before harvesting.

Preparation and Subfractionation of Mitochondria—Yeast was grown in YP broth supplemented with 2% ethanol, and mitochondria were isolated (21). Integral membrane proteins were separated from soluble and peripheral proteins by extraction of mitochondria (1 mg of protein/ml) with 0.1 M sodium carbonate for 30 min at 0 °C and centrifugation (226,000 × *g* for 1 h at 2 °C). The pellet contained integral membrane proteins, and the supernatant contained peripheral and soluble proteins (22).

Mitochondria were resuspended in buffer (10 mM K^+-MOPS, pH 7.2, 0.25 M sucrose, 5 mM EDTA, 1 mM *o*-phenanthroline, and 0.2 M KCl; final protein concentration, 1 mg/ml) and extracted with digitonin (23). The solutions were mixed and placed on ice for 1 min. They were diluted with 4 volumes of buffer and centrifuged (100,000 × *g* for 10 min). The soluble proteins were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and Western blotting with a rabbit antibody against recombinant OAC (24).

Swelling of Yeast Mitochondria—Swelling of mitochondria in an isoosmotic solution of an ammonium salt indicates that an anion permeates the mitochondrion with protons (or in exchange for hydroxyl ions) (25). Molecular ammonia is protonated inside the mitochondria, and the pH gradient collapses. The rate of swelling of mitochondria was monitored by the decrease in A_{546} (7). Yeast mitochondria (100 μg of protein) were added to 1 ml of various ammonium salts (120 mM), 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 μM rotenone, and 0.1 μM antimycin.

Membrane Potentials—The membrane potential of mitochondria was assessed from the fluorescence changes of the voltage-sensitive dye $DiSC_3(5)$ (26).

RESULTS

Bacterial Expression of OAC—Yeast OAC was overexpressed in *E. coli* C0214(DE3) at high levels (Fig. 1, *lane 4*). Its apparent molecular mass was about 37 kDa (calculated value including the initiator methionine, 35,165 Da). The purified inclusion bodies gave rise a single band on SDS-PAGE gels with the same apparent molecular mass (Fig. 1, *lane 5*). It was detected neither in bacteria harvested immediately before induction of expression (*lane 2*) nor in cells harvested after induction but lacking the OAC coding sequence in the expression vector (*lane 3*). The N-terminal sequence of the 37-kDa protein, SSDNSKQDKQIE, is identical to residues 2–13 of OAC. About 60 mg of purified protein (Fig. 1, *lane 5*) were obtained per liter of culture.

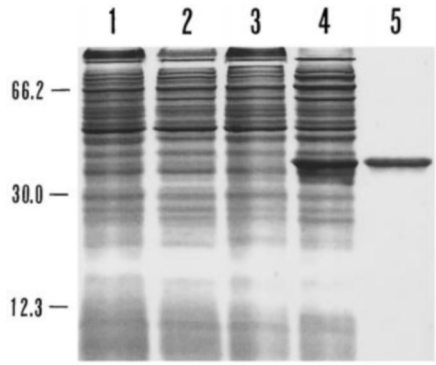

FIG. 1. **Expression of yeast OAC in *E. coli*.** Proteins were separated by SDS-PAGE and stained with Coomassie Blue dye. The positions of markers (bovine serum albumin, carbonic anhydrase, and cytochrome *c*) are shown on the *left*. *Lanes 1–4, E. coli* C0214(DE3) containing the expression vector, without (*lanes 1* and *3*), and with the coding sequence of OAC (*lanes 2* and *4*). Samples were taken at the time of induction (*lanes 1* and *2*) and 5 h later (*lanes 3* and *4*). The same number of bacteria was analyzed in each sample. *Lane 5,* purified OAC (15 μg) originating from bacteria shown in *lane 4*.

Functional Characterization of Recombinant OAC—The reconstituted protein catalyzed an active [^{14}C]malonate/malonate exchange and a more active [^{35}S]sulfate/sulfate exchange (Table I) that were inhibited by a mixture of *p*-chloromercuribenzene sulfonate and bathophenanthroline. Homoexchange activities more than 1 order of magnitude lower than these activities were measured for malate, oxoglutarate, and phosphate. No homoexchange activities were detected with OAC that had been boiled before incorporation into liposomes or by reconstitution of sarkosyl-solubilized material from bacterial cells either lacking the expression vector for OAC or harvested immediately before induction of overexpression. The proteoliposomes did not catalyze homoexchanges for fumarate, citrate, glutamate, aspartate, glutamine, carnitine, ornithine, and ADP (external concentration, 1 mM; internal concentration, 10 mM) (Table I).

In Fig. 2*A*, the time-courses are compared of uptake by proteoliposomes of 1 mM [^{14}C]malonate measured either as uniport (in the absence of internal malonate) or as exchange (in the presence of 10 mM malonate). Both data sets fitted a first-order rate equation with rate constants (k) for the exchange and the uniport reactions of 0.15 and 0.14 min^{-1}, respectively. Maximum uptake was approached after 30 min. The corresponding values at infinite time were 1.46 and 0.16 μmol/mg. The ratio of maximal substrate uptake by the exchange and by the uniport was 9.1, in agreement with the expected value of 10 from the intraliposomal concentrations at equilibrium (1 and 10 mM for uniport and exchange, respectively). The initial rates of malonate uptake (the product of k and intraliposomal quantity of malonate taken up at equilibrium (18)) were 213 and 23 μmol/min/g of protein for the exchange and uniport reactions, respectively. The addition of 10 mM unlabeled malonate after 30 min incubation, when radioactive uptake by the proteoliposomes had approached equilibrium, caused an extensive efflux of radiolabeled malonate from both malonate-loaded and unloaded proteoliposomes (Fig. 2*A*). This efflux indicates that the [^{14}C]malonate taken up by uniport is released by exchange for externally added substrate.

Upon addition to liposomes that had been preincubated for 30 min with 1 mM [^{14}C]malonate in the absence of internal substrate of 10 mM malonate, oxaloacetate, or sulfate, an extensive efflux of intraliposomal radioactivity was observed (Fig. 2*B*). Upon addition of malate, oxoglutarate, and phosphate, very little efflux was detected, and addition of fumarate had no

TABLE I
Rates of homo-exchanges of various substrates in proteoliposomes containing recombinant yeast OAC

Transport was initiated by adding radioactive substrate (final concentration, 1 mM) to proteoliposomes preloaded internally with the same substrate (concentration, 10 mM). Similar results were obtained in three independent experiments.

	Substrate transport
	μmol/min/g protein
Malonate	222
L-Malate	25
Fumarate	0
Sulfate	596
Phosphate	10
Oxoglutarate	12
Citrate	0
Glutamate	0
Aspartate	0
Glutamine	0
Ornithine	0
Carnitine	0
ADP	0

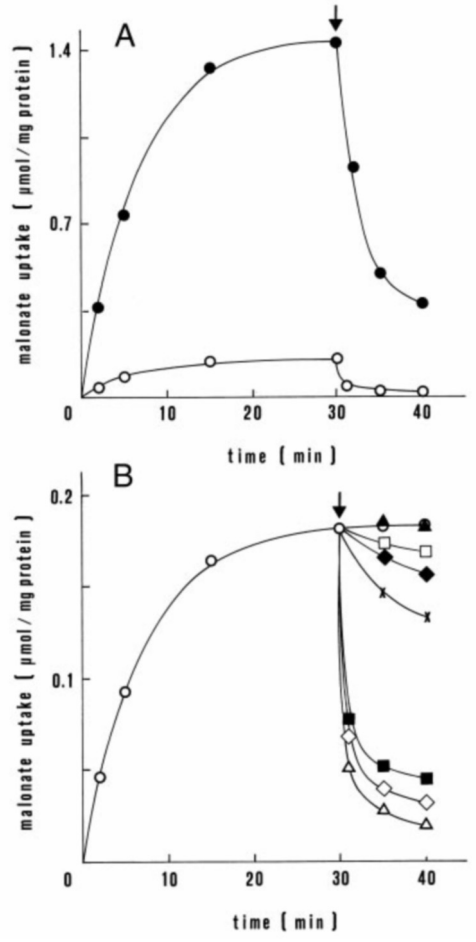

FIG. 2. **Time-course of [^{14}C]malonate uptake into proteoliposomes and its efflux after addition of an excess of unlabeled substrates.** *A,* 1 mM [^{14}C]malonate was added to unloaded proteoliposomes (uniport (○)), or to proteoliposomes loaded with 10 mM malonate (exchange (●)). The *arrow* indicates the addition of 10 mM nonradioactive malonate. *B,* 1 mM [^{14}C]malonate was added to unloaded proteoliposomes. The *arrow* indicates the addition of 10 mM nonradioactive malonate (△), oxaloacetate (■), sulfate (◇), L-malate (×), oxoglutarate (♦), phosphate (□), or fumarate (▲).

effect. These results indicate clearly that oxaloacetate, sulfate, and malonate are good substrates for reconstituted OAC.

The substrate specificity of reconstituted OAC was investigated further by measuring the uptake of [^{14}C]malonate and

TABLE II
Dependence on internal substrate of the transport properties of proteoliposomes containing recombinant yeast OAC

Proteoliposomes were preloaded internally with various substrates (concentration, 10 mM). Transport was started by the external addition of [^{14}C]malonate or [^{35}S]sulfate (final concentrations, 0.5 mM) and terminated after 5 min. Similar results were obtained in three independent experiments.

Internal substrate (10 mM)	Substrate transport	
	[^{14}C]Malonate	[^{35}S]Sulfate
	μmol/5 min/g protein	
None (Cl^- present)	129	116
Malonate	883	1252
Sulfate	947	1597
Thiosulfate	964	1488
Oxaloacetate	956	1305
L-Malate	296	621
Succinate	214	263
Oxoglutarate	258	407
Phosphate	253	352
Fumarate	145	198
Pyruvate	123	107
Aspartate	92	104
Glutamate	105	98
Citrate	137	152
ADP	114	125
Ornithine	138	146
Glutamine	127	109
Carnitine	130	182

[^{35}S]sulfate into proteoliposomes that had been preloaded with various substrates (Table II). The highest activities were observed in the presence of internal sulfate, thiosulfate, oxaloacetate, and malonate. Lower activity was found in the presence of internal malate, succinate, oxoglutarate, and phosphate. Virtually no exchange was detected with internal fumarate, pyruvate, aspartate, glutamate, citrate, ADP, ornithine, glutamine, and carnitine. The residual activity in the presence of these substrates was virtually the same as the activity observed without internal substrate. Therefore, the substrate specificity of the OAC protein is confined essentially to sulfate, thiosulfate, and the physiologically important dicarboxylate oxaloacetate.

The uptake of [^{14}C]malonate by proteoliposomes was inhibited by sulfydryl reagents (mersalyl, *p*-chloromercuribenzene sulfonate, and *N*-ethylmaleimide), by pyridoxal 5-phosphate, and by bathophenanthroline (Table III). The impermeable dicarboxylate analogues butylmalonate and benzylmalonate also inhibited the transport activity strongly. In contrast, carboxyatractyloside, 1,2,3-benzenetricarboxylate and α-cyanocinnamate, inhibitors of other characterized mitochondrial carriers, had no effect on the activities of reconstituted OAC. The ability of nonradioactive potential substrates to inhibit the uptake of [^{14}C]malonate was also examined (Table III). Malonate transport was prevented by external addition of malonate, sulfate, thiosulfate, and oxaloacetate, substrates that are transported by OAC (Tables I and II and Fig. 2*B*). To a lesser extent, poor substrates such as malate, phosphate, oxoglutarate, and succinate inhibited the OAC-catalyzed transport of [^{14}C]malonate.

Kinetic Characteristics of Recombinant OAC—The K_m and V_{max} values for malonate uptake by unloaded proteoliposomes (measured as uniport at 25 °C), from a typical experiment (Fig. 3) were 0.11 mM and 25 μmol/min/g of protein, respectively. The average values of K_m and V_{max} from 18 experiments were 0.1 ± 0.01 mM and 22 ± 6 μmol/min/g of protein, respectively. Oxaloacetate and sulfate inhibited malonate uptake competitively (Fig. 3). All of the compounds summarized in Table IV

TABLE III

Effect of inhibitors and externally added substrates on the uptake of [^{14}C]malonate into proteoliposomes reconstituted with recombinant OAC

Transport was started by adding [^{14}C]malonate (final concentration, 0.1 mM) to proteoliposomes containing 10 mM NaCl and no substrate. Thiol reagents, pyridoxal 5′-phosphate, carboxyatractyloside and α-cyanocinnamate were added 3 min before the labeled substrate; the other inhibitors and external substrates were added together with [^{14}C]malonate. All inhibitors and substrates were present at final concentrations of 2 mM, except for organic mercurials (0.001 mM), carboxyatractyloside and α-cyanocinnamate (0.1 mM), and pyridoxal 5′-phosphate (10 mM). The control values for uninhibited malonate uptake were 12.4 (experiment 1) and 14.0 (experiment 2) μmol/min per g of protein. Similar results were obtained in three independent experiments.

Reagents	Inhibition
	%
Experiment 1	
Mersalyl	99
p-Chloromercuriphenylsulfonate	99
N-Ethylmaleimide	68
Bathophenanthroline	85
Pyridoxal 5′-phosphate	84
Butylmalonate	93
Benzylmalonate	88
1,2,3-Benzenetricarboxylate	12
Carboxyatractyloside	4
α-Cyanocinnamate	7
Experiment 2	
Malonate	96
Sulfate	87
Thiosulfate	82
Sulfite	85
Oxaloacetate	84
Malate	56
Phosphate	46
Oxoglutarate	30
Succinate	23
Pyruvate	6
Citrate	7
ADP	4
Carnitine	10
Ornithine	7

are competitive inhibitors with respect to malonate, because they were found to increase the apparent K_m without changing the V_{max} of malonate uptake (not shown). The K_i value of L-malate is significantly lower than the respective K_m value (> 6 mM) as determined from homoexchange rate measurements, indicating that although malate is poorly transported by OAC, it has a high affinity for its substrate-binding site.

Subcellular Localization of OAC—A single immunoreactive band with an apparent molecular mass of about 36.2 kDa was detected in the wild-type mitochondria, but not in mitochondria from the Δ-OAC1 strain (Fig. 4). The calculated mass of OAC including the initiator methionine is 35,165 Da. The contents of succinate-fumarate, ADP/ATP, phosphate, and the dicarboxylate carriers detected with specific antibodies were essentially the same in wild-type and Δ-*OAC1* mitochondria. Therefore, the absence of OAC from the Δ-*OAC1* strain does not reduce expression of other carriers.

The submitochondrial location of OAC was examined by separation of soluble and peripheral proteins from integral membrane proteins of wild-type mitochondria by carbonate treatment (Fig. 5, *A* and *B*). OAC remained in the membrane protein fraction, as did the ADP/ATP carrier and Tom40 (marker proteins of inner and outer mitochondrial membranes, respectively), but the intermembrane space protein cytochrome b_2 and the matrix protein hsp70 were in the soluble and peripheral protein fraction. Therefore, OAC is an integral mitochondrial membrane protein.

Both OAC and the ADP/ATP carrier were solubilized from wild-type mitochondria at the same concentration of digitonin,

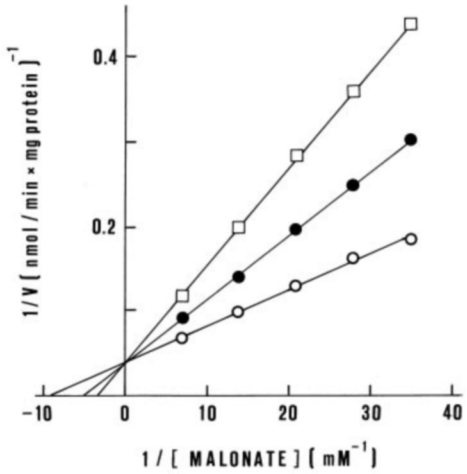

FIG. 3. **Dependence of the rate of malonate uptake into proteoliposomes on the external malonate concentration and competitive inhibition by oxaloacetate and sulfate.** The [^{14}C]malonate was added to unloaded proteoliposomes at the indicated concentrations. Except in the control (○), 0.2 mM oxaloacetate (●) or 0.12 mM sulfate (□) was added simultaneously with [^{14}C]malonate.

TABLE IV

Competition with [^{14}C]malonate uptake in proteoliposomes containing recombinant yeast OAC

The values were calculated from double reciprocal plots of the rate of [^{14}C]malonate uptake *versus* substrate concentrations. For experimental conditions, see Fig. 3. The competing substrates were added at the appropriate concentrations simultaneously with [^{14}C]malonate. The data represent the means ± S.D. of at least three different experiments.

Substrate	K_i
	mM
Sulfate	0.07 ± 0.02
Thiosulfate	0.17 ± 0.02
Sulfite	0.06 ± 0.01
Oxaloacetate	0.25 ± 0.09
Malate	1.1 ± 0.2
Oxoglutarate	3.4 ± 0.4
Phosphate	2.0 ± 0.4

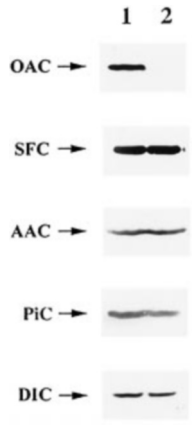

FIG. 4. **Immunoblot analysis of OAC in yeast mitochondria.** *Lanes 1* and *2*, wild-type and Δ*OAC1* mitochondria (25 μg of protein), respectively, separated by SDS-PAGE, transferred to nitrocellulose, and detected with specific antibodies for the OAC and for the carriers for succinate/fumarate (*SFC*), ADP/ATP (*AAC*), phosphate (*PiC*), and dicarboxylate (*DIC*).

which was greater than that required to solubilize the outer membrane protein Tom40 (Fig. 5*C*). Therefore, OAC is an integral protein of the inner mitochondrial membrane.

Mitochondria Lacking OAC Are Impaired in Uptake of Sulfate, Oxaloacetate, and Malonate—Wild-type and Δ-*OAC1* cells

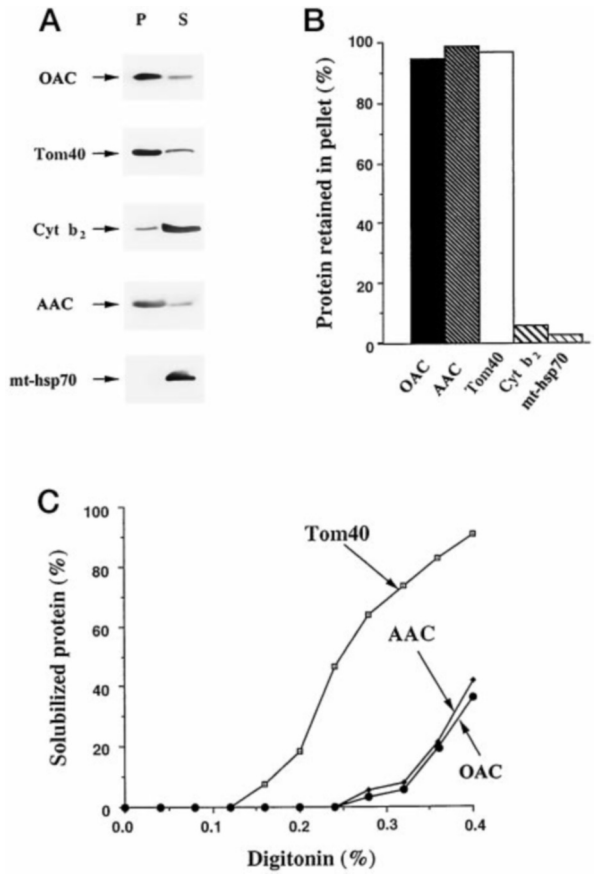

FIG. 5. **Submitochondrial localization of OAC.** *A* and *B,* analysis by SDS-PAGE and Western blotting of soluble (*S*) and peripheral (*P*) proteins and intrinsic membrane proteins from yeast mitochondria blotted with antisera directed against the OAC, ADP/ATP carrier (*AAC*) (inner membrane component), Tom40 (outer membrane component), cytochrome b_2 (*Cyt b_2*) (intermembrane space marker), and mitochondrial hsp70 (*mt-Hsp70*) (matrix protein). *C,* digitonin extracts (0–0.4% detergent) of yeast mitochondria separated by SDS-PAGE. The solubilization of OAC, AAC, and Tom40 was estimated by immunoblotting. The protein content of the unextracted membrane fraction was taken as 100%.

had similar growth characteristics in complete synthetic medium and in YP broth, but their mitochondria differed in several respects. For example, wild-type mitochondria swelled in 120 mM solutions of ammonium salts of malonate, oxaloacetate, sulfate, and thiosulfate (Fig. 6), whereas mitochondria from the Δ-*OAC1* strain did not do so appreciably. Both mitochondria swelled in ammonium phosphate, indicating the presence of an active phosphate carrier, but neither did in the ammonium salts of malate (mainly transported by the dicarboxylate carrier; swelling requires a catalytic trace of phosphate), fumarate (transported by the succinate-fumarate carrier), or chloride (impermeant anion). Therefore, the absence of OAC does not affect the integrity of the mitochondria and the activity of other carriers.

The validity of the swelling method was confirmed by control experiments on mitochondria from a strain in which the gene for the dicarboxylate carrier has been disrupted (Δ*DIC1*). They swelled in the presence of the ammonium salts of low affinity substrates of the dicarboxylate carrier (oxaloacetate, malonate, sulfate, and thiosulfate) at a very similar rate to wild-type mitochondria (Fig. 6), but not in the presence of ammonium malate plus phosphate, in agreement with the absence of the dicarboxylate carrier (7). When the external pH was decreased from 8.0–5.9, the rate of swelling in ammonium sulfate increased strongly (Fig. 7*A*), confirming the dependence of the sulfate/H^+ influx on ΔpH. Similar results were obtained with ammonium oxaloacetate and ammonium malonate (data not shown).

Further evidence that the influx into the mitochondrial matrix of sulfate, oxaloacetate, and malonate is carrier-mediated came from the study of the effects of inhibitors of OAC on swelling. In isoosmotic ammonium sulfate, swelling of wild-type mitochondria is strongly inhibited by 20 mM bathophenanthroline and abolished almost completely by 0.2 mM mersalyl or *p*-chloromercuribenzenesulfonic acid (Fig. 7*B*). Organic mercurials and bathophenanthroline inhibited mitochondrial swelling in ammonium salts of oxaloacetate and malonate (data not shown). Therefore, mitochondrial swelling and activity of recombinant OAC are inhibited by the same reagents.

Finally, the membrane potential ($\Delta\psi$) of wild-type and Δ-*OAC1* mitochondria were compared with the fluorescence dye $DiSC_3(5)$ (27). The fluorescence difference of the mitochondria and substrates, before and after the addition of valinomycin (in the presence of K^+), was virtually the same in wild-type and Δ-*OAC1* mitochondria (data not shown). Therefore, deletion of the *OAC1* gene does not influence the coupling properties of the inner mitochondrial membrane.

Functional Expression of OAC in the Deletion Strain—OAC was expressed about 30 times higher in mitochondria in the *OAC1*-pYeDP60 strain than in wild-type mitochondria. Increased activity was evident from rapid mitochondrial swelling in ammonium sulfate or ammonium malonate (Fig. 6), and from increased uptake into proteoliposomes reconstituted with a digitonin extract of those mitochondria of [^{35}S]sulfate in exchange for sulfate, oxaloacetate, or malonate (Fig. 8). The sulfate/sulfate, sulfate/oxaloacetate, and sulfate/malonate exchange activities were several times higher than in Δ-OAC1 and wild-type mitochondria, whereas the sulfate/phosphate exchange (mainly the dicarboxylate carrier) was little increased in *OAC1*-pYeDP60 mitochondria. Similarly, the [^{14}C]malate/phosphate exchange (the defining reaction of the dicarboxylate carrier) was virtually the same in wild-type and *OAC1*-pYeDP60 mitochondria (not shown). Therefore, the large difference between the activities of the reactions catalyzed by OAC in Δ-*OAC1* and *OAC1*-pYeDP60 mitochondria strongly supports the conclusion that OAC functions as a transporter for oxaloacetate and sulfate.

DISCUSSION

Identification of Carriers of Unknown Function—The identification of yeast OAC as an oxaloacetate and sulfate carrier provides a further demonstration of the utility of the procedure for overexpressing the mitochondrial carriers in *E. coli* and of reconstituting the recombinant proteins in liposomes in order to study their transport properties. This procedure, originally developed with the bovine oxoglutarate/malate carrier (15), has been applied subsequently to the independently identified phosphate (17, 28) and carnitine (29) carriers and has allowed the yeast carriers for citrate (30), dicarboxylate (5), ornithine (31), and succinate-fumarate (6) to be identified.

The use of *S. cerevisiae* strains containing deleted carrier genes has provided evidence that the dicarboxylate and ornithine carriers operate with the same transport properties *in vivo* as *in vitro* (7, 32).

Kinetic Properties of OAC—Four observations demonstrate that reconstituted OAC catalyzes both exchange and uniport. First, the ratio between malonate taken up by exchange and uniport at equilibrium agrees with the expected value if all of the carrier-loaded liposomes catalyze both exchange and uniport of malonate. Second, addition of unlabeled substrates releases nearly all [^{14}C]malonate taken up by uniport. Third, both exchange and uniport are inhibited by low concentrations

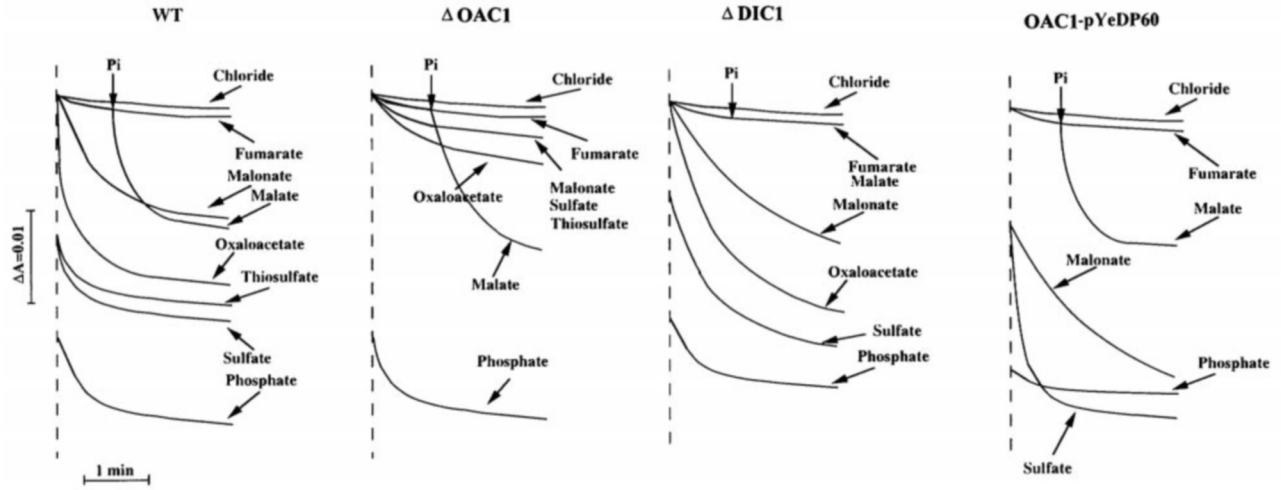

FIG. 6. **Swelling of wild-type, Δ*OAC1*, Δ*DIC1*, and *OAC1*-pYeDP60 yeast mitochondria in isoosmotic ammonium solutions of various anions.** Mitochondria (0.1 mg of protein) were suspended at 25 °C in a solution containing ammonium salts (120 mM) of the indicated anions, 20 mM Tris, pH 7.4, 1 mM EDTA, 0.1 μM antimycin, and 5 μM rotenone in a final volume of 1 ml. The turbidity changes of the mitochondrial suspensions were recorded at 546 nm. The *arrows* indicate the addition of 5 mM potassium phosphate (*Pi*).

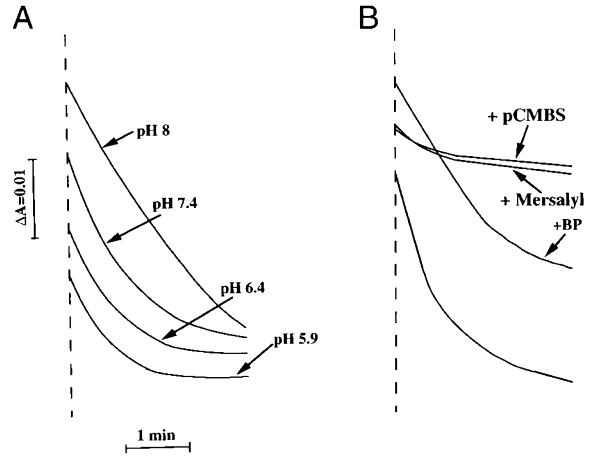

FIG. 7. **Effect of pH and inhibitors on the swelling of yeast mitochondria in ammonium sulfate.** Mitochondria (0.1 mg of protein) were suspended at 25 °C in a solution containing 120 mM ammonium sulfate, 20 mM Tris, pH 7.4, 1 mM EDTA, 0.1 μM antimycin, and 5 μM rotenone in a final volume of 1 ml. Turbidity changes were recorded at 546 nm. *A,* effect of pH changes. *B,* effect of inhibitors. The solution contained 20 mM bathophenanthroline (*BP*), 0.2 mM mersalyl, or 0.2 mM *p*-chloromercuribenzenesulfonic acid (*pCMBS*).

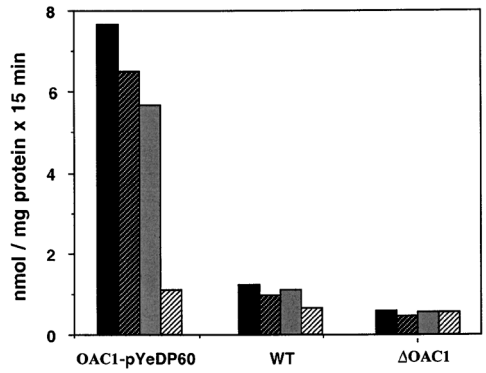

FIG. 8. **Sulfate exchange activities in liposomes reconstituted with mitochondrial extracts from the Δ*OAC1* strain, the parental strain, and the deletion strain transformed with the OAC1-pYeDP60 plasmid.** Mitochondria (0.4 mg of protein/ml) were solubilized in 1% digitonin, 50 mM NaCl, 1 mM EDTA, and 10 mM PIPES (pH 7.0) for 20 min at 0 °C and centrifuged (138,000 × *g* for 30 min). Supernatants (about 6 μg of protein) were reconstituted into liposomes, preloaded with 20 mM sulfate (*black columns*), oxaloacetate (*dark hatched columns*), malonate (*gray columns*), or phosphate (*light hatched columns*). Transport was started by adding 0.1 mM [^{35}S]sulfate and terminated after 15 min.

of bathophenanthroline and mercurials. Fourth, the first-order rate constant for uniport is practically the same as that measured for the exchange. However, the exchange rate is much faster than the uniport rate, and so the OAC resembles other reconstituted mitochondrial carriers (phosphate, carnitine, and ornithine) (31, 33, 34). The physiologically important reaction for the phosphate carrier is the uniport (P_i/H^+ symport), whereas for the carnitine and the ornithine carriers, both exchange and unidirectional reactions are important. Because the apparent V_{max} depends on the experimental conditions of the reconstituted system, and the amount of active carrier molecules present in the proteoliposomes is not known, the transport rates measured in the reconstituted system may differ substantially from the activities *in vivo*.

The substrate specificity of the OAC is distinct from those of the dicarboxylate and oxoglutarate carriers (the principal substrates of which are malate and phosphate, and oxoglutarate and malate, respectively), or from any other characterized mitochondrial carrier. Nonetheless, L-malate, phosphate, and oxoglutarate are transported to some extent by the OAC protein, and so its specificity overlaps with those of the dicarboxylate and the oxoglutarate carriers. When homoexchanges are measured (Table I), the rate is less than ½0 of that of the sulfate/sulfate exchange. However, a low rate of transport takes place when the exchange of intraliposomal malate, oxoglutarate, or phosphate at high concentrations (10 mM) is measured against sulfate and malonate, which are good substrates for OAC (Table II). The inhibition constants of oxoglutarate, phosphate, and expecially malate on the rate of malonate uptake (Table III) also indicate a rather significant affinity for OAC. However, the half-saturation transport constant for malate (>6 mM) is much higher than its K_i, clearly showing that malate binds to the substrate-binding site of OAC, although it is poorly transported. Given the rather close similarity of the sequences of the OAC and the dicarboxylate and the oxoglutarate carriers, this is expected.

Transport Mechanism—The swelling properties of the wild-type yeast mitochondria were strikingly different from those of rat liver. The former swell in ammonium oxaloacetate, sulfate,

thiosulfate, and malonate, whereas the latter do not swell either in ammonium oxaloacetate,[3] or in ammonium sulfate, thiosulfate, or malonate (35, 36). Therefore, oxaloacetate, sulfate, thiosulfate, and malonate are transported into the yeast mitochondrial matrix with protons (or in exchange for hydroxyl ion) (25), and the ΔpH component of the protonmotive force drives their entry into the matrix (37). This transport mechanism is consistent with the ability of the recombinant OAC to catalyze influx of its substrates into proteoliposomes in the absence of internal substrate.

Physiological Roles of OAC—As the *S. cerevisiae* pyruvate carboxylase is cytoplasmic, one physiological role of OAC is probably to catalyze uptake of oxaloacetate into mitochondria, a role supported by the higher transcript level in synthetic medium than in rich medium (13). The *OAC1* gene is not essential for growth of *S. cerevisiae*, and it is likely that in the absence of OAC, oxaloacetate is converted to malate in the cytoplasm, and malate enters the mitochondrion via the dicarboxylate carrier in exchange for phosphate. A role in anaplerosis for both the OAC and the dicarboxylate carrier (7) is consistent with the failure of a yeast strain with both carrier genes deleted to grow on nonfermentable carbon sources (not shown).

Another possible role for the OAC may be to transfer reducing equivalents from the mitochondrial matrix to the cytosol by catalyzing a malate/oxaloacetate exchange when the intramitochondrial concentrations of NADH and malate are high. A malate-oxaloacetate shuttle has been proposed to operate in mammalian and plant mitochondria (38–40).

The OAC and the dicarboxylate carrier are the only proteins with significant sequence homology to the bovine oxoglutarate carrier, and so it is unlikely that *S. cerevisiae* has an oxoglutarate/malate carrier. One important difference between animal and yeast mitochondria is the mechanism for reoxidation of cytosolic NADH. In animals, the oxoglutarate/malate and glutamate/aspartate carriers produce a net transport of reducing equivalents from the cytosol to the mitochondrial matrix by the aspartate-malate shuttle. In *S. cerevisiae* and in plants, cytosolic NADH is oxidized on the exterior of the inner mitochondrial membrane, making the presence of the oxoglutarate/malate carrier unnecessary. Therefore, it is possible that OAC and the oxoglutarate carrier have evolved from a close common ancestor.

Transport of sulfate and thiosulfate may be important in sulfur metabolism. In mammals, the conversion of thiosulfate to sulfite is catalyzed by mitochondrial thiosulfate sulfurtransferase (rhodanase) and thiosulfate reductase (41, 42). A "sulfate uniport by a transport system sensitive to *N*-ethylmaleimide that shares the properties of the phosphate carrier" (*i.e.* electroneutral H^+-compensated) in rat mitochondria (43) is the only current evidence for a mammalian OAC.

[3] L. Palmieri, A. Vozza, G. Agrimi, V. De Marco, M. J. Runswick, F. Palmieri, and J. E. Walker, unpublished observations.

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