Identification of the Yeast Mitochondrial Transporter for Oxaloacetate and Sulfate

Luigi Palmieri, Angelo Vozza, Gennaro Agrimi, Valeria De Marco, Michael J. Runswick, Ferdinando Palmieri and John E. Walker

From the Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, Via Orabona 4, 70125 Bari, Italy and the Medical Research Council, Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2DH, United Kingdom

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 thiocarbonate. 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, thiocarbonate, and malonate. Mitochondria from wild-type cells swelled in isoosmotic solutions of ammonium salts of oxaloacetate, sulfate, thiocarbonate, and malonate, indicating that these anions are cotransported with protons. Overexpression of OAC in the deletion strain increased greatly the [35S]sulfate/sulfamate and [32P]phosphate/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cerevisiae has two principal pathways for replenishing the intermediates of the Krebs cycle that have been withdrawn for biosynthesis.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. These two cytosolic isozymes of pyruvate carboxylase. Mammalian been withdrawn for biosynthesis. They are the glyoxylate cycle

EXPERIMENTAL PROCEDURES

Materials—[2-14C]Malonic acid, a-[1,14C]-ketoglutaric acid, [1,5-14C]citric acid, [1-14C]glutamic acid, [1-14C]aspartic acid, [1-14C]glutamine and [8-14C]adenosine 5′-diphosphate trisodium salt were supplied by NEN Life Science Products. L-[2,3-3H]ornithine were from Sigma. Egg yolk phospholipids were purchased from Fluka, and cardioplin 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 OAC 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 XL). Additional NdeI and HindIII 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 in 15% 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 nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AJ238698.

* 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 Scientific 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.

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).

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org

22184
Mitochondrial Oxaloacetate Carrier

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-chloromercuribenzoate sulphonate 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 AG1-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 ade2-101 his3-Δ200 leu2-3,112 ura3-52 trp1-Δ3 63 lys2-b8-501 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 fermentable 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 mM sucrose, 5 mM EDTA, 1 mM o-phenanthroline, and 0.2 mM 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 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 mitochondrial membrane 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 mM rotenone, and 0.1 μM antimycin.

Membrane Potentials—The membrane potential of mitochondria was assessed from the fluorescence changes of the voltage-sensitive dye DSC3(5) (126).

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, SSDNSKQKQIE, is identical to residues 2–13 of OAC. About 60 mg of purified protein (Fig. 1, lane 5) were obtained per liter of culture.

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-chloromercuribenzenesulfonyl fluoride and bathophenanthroline. Homoe massa exchange activities more than 1 order of magnitude lower than these activities were measured for malate, oxoglutarate, and phosphate. No homoe massa exchange activities were detected with OAC that had been boiled before incorporation into liposomes or by reconstitution of sarcosyl-solubilized material from bacterial cells either lacking the expression vector for OAC or harvested immediately before induction of overexpression. The proteoliposomes did not catalyze homoe massa exchanges for fumarate, citrate, glutamate, aspartate, glutamine, carnitine, ornithine, and ADP (external concentration, 1 mM; internal concentration, 10 mM) (Table I).

In Fig. 2A, 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 reaction 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. 2A). 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 radiolabel was observed (Fig. 2B). Upon addition of malate, oxoglutarate, and phosphate, very little efflux was detected, and addition of fumarate had no

![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.](image-url)
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 \([^{35}S]\)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, \(\text{p}-\text{chloromercuribenzenesulfonate}, \text{and } \text{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 \(\alpha\)-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. 2B). 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 \(V_{\text{max}}\) 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 \(\mu\text{mol/min/g of protein}\), respectively. The average values of \(K_m\) and \(V_{\text{max}}\) from 18 experiments were 0.11 ± 0.01 mM and 22 ± 6 \(\mu\text{mol/min/g of protein}\), respectively. Oxaloacetate and sulfate inhibited malonate uptake competitively (Fig. 3).
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 homoeexchange 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 \( \Delta \text{OAC1} \) 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 \( \Delta \text{OAC1} \) mitochondria. Therefore, the absence of OAC from the \( \Delta \text{OAC1} \) strain does not reduce expression of other carriers.

The sub mitochondrial 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, which was greater than that required to solubilize the outer membrane protein Tom40 (Fig. 5C). 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 \( \Delta \text{OAC1} \) cells

**TABLE III**

| Reagents Inhibition | % |
|---------------------|---|
| Experiment 1        |   |
| Mersalyl            | 99|
| p-Chloromercuriphenylsulfonate | 99|
| N-Ethylmaleimide    | 68|
| Bathophenanthroline | 85|
| Pyridoxal 5'-phosphate | 84|
| Butyrlmalonate      | 93|
| Benzylmalonate      | 88|
| 1,2,3-Benzenetricarboxylate | 12|
| Carboxyatractyloside | 4|
| \( \alpha \)-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|

**TABLE IV**

| 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. 3.** Dependence of the rate of malonate uptake into proteoliposomes on the external malonate concentration and competitive inhibition by oxaloacetate and sulfate. The [\( ^{14} \text{C} \)]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 [\( ^{14} \text{C} \)]malonate.

**FIG. 4.** Immunoblot analysis of OAC in yeast mitochondria. Lanes 1 and 2, wild-type and \( \Delta \text{OAC1} \) 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).

**FIG. 5.** Dependence of the rate of malonate uptake into proteoliposomes reconstituted with recombinant yeast OAC

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

**Mitochondrial Oxaloacetate Carrier**
Mitochondrial Oxaloacetate Carrier

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, in agreement with the absence of OAC carriers. The protein content of the unextracted membrane fraction was taken as 100%.

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. 7C). 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 (Δψ) of wild-type and Δ-OAC1 mitochondria were compared with the fluorescence dye DiSC3(5) (27). The fluorescence difference of the mitochondrial 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 [35S]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 [14C]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 [14C]malonate taken up by uniport. Third, both exchange and uniport are inhibited by low concentrations of sulfate/H⁺ influx on ΔpH. Similar results were obtained with ammonium oxaloacetate and ammonium malonate (data not shown).

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 b₅ (Cyt b₅) (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%.
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 (Pi/H\textsuperscript{+} symport), whereas for the carnitine and ornithine carriers, both exchange and unidirectional reactions are important. Because the apparent V\textsubscript{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 1/20 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 especially 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 (\(K_{\text{m}}\)) is much higher than its \(K_{\text{i}}\), 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 \(\Delta p\text{H}\) component of the protonotive 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 anaerobiosis for both the OAC and the dicarboxylate carrier (7) is consistent with the presence 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 sulfurylartransferase (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.