The Mitochondrial Ornithine Transporter

BACTERIAL EXPRESSION, RECONSTITUTION, FUNCTIONAL CHARACTERIZATION, AND TISSUE DISTRIBUTION OF TWO HUMAN ISOFORMS*

Received for publication, March 6, 2003, and in revised form, June 13, 2003
Published, JBC Papers in Press, June 13, 2003, DOI 10.1074/jbc.M302317200

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Two isoforms of the human ornithine carrier, ORC1 and ORC2, have been identified by overexpression of the proteins in bacteria and by study of the transport properties of the purified proteins reconstituted into liposomes. Both transport L-isomers of ornithine, lysine, arginine, and citrulline by exchange and by unidirectional mechanisms, and they are inactivated by the same inhibitors. ORC2 has a broader specificity than ORC1, and L- and D-histidine, L-homoarginine, and D-isomers of ornithine, lysine, and ornithine are all substrates. Both proteins are expressed in a wide range of human tissues, but ORC1 is the predominant form. The highest levels of expression of both isoforms are in the liver. Five mutant forms of ORC1 associated with the human disease hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome were also made. The mutations abolish the transport properties of the protein. In patients with hyperornithinemia-hyperammonemia-homocitrullinuria, isoform ORC2 is unmodified, and its presence compensates partially for defective ORC1.

Rat liver mitochondria contain an ornithine-citrulline transport protein, often known as the ornithine carrier (ORC)1 (1–5). The reconstituted purified protein is highly active in ornithine-citrulline exchange and is somewhat less active in unidirectional transport of ornithine. It transports lysine and arginine also but does not transport histidine. The positive charges of ornithine and lysine are compensated by cotransport of a proton with citrulline in exchange. Its affinity for ornithine is lower than for other substrates, and under saturating internal concentrations it is not dependent on the nature of the counter-substrate. The exchange reaction operates by a simultaneous (sequential) mechanism, and the unidirectional transport of ornithine or lysine (but not of citrulline) is compensated by a proton. The exchange of cytosolic ornithine for matrix citrulline is part of the urea cycle (1, 3, 6), and the ornithine–H+ exchange also has an important role in the catabolism of excess arginine and the biosynthesis of polyamines (4).

In Saccharomyces cerevisiae, the ornithine carrier is encoded by ARG11/ORT1, and its identity has been established by overexpression in bacteria, reconstitution, and study of its transport properties (7). Subsequently, the gene for the human mitochondrial ornithine carrier, which is defective in the hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (8), was identified by homology with the yeast sequence, and its function as an ORC was inferred from its ability to complement the defect of HHH fibroblasts by incorporating radioactive ornithine into cellular protein. The HHH phenotype is milder than those associated with a deficiency of an enzyme in the urea cycle, and it has been suggested that carriers with other functions can compensate for the defective protein by transporting ornithine (8).

As described below, the human and mouse genomes contain a spliced pseudogene that in man encodes a second isoform, ORC2 (9). The isoforms are expressed differently in various human tissues. Both were overexpressed in Escherichia coli. The purified proteins were reconstituted into liposomes, and their transport properties were characterized. The recombinant proteins have different substrate specificities, transport affinities (Km), and specific activities (Vmax). Five independent mutations associated with HHH were introduced singly into the isoform ORC1. They affected its transport properties severely. In the same patients with HHH, ORC2 is unaffected, and therefore, it is likely that it compensates for defective ORC1.

EXPERIMENTAL PROCEDURES

Sequence Search and Analysis—Data bases were screened with the sequence of ORC1 (GenBank™/EBI accession number AF112986, gene name SLC25A15) with BLASTP and TBLASTN. The amino acid sequences were aligned with ClustalW (version 1.7).

Construction of Expression Plasmids—The coding sequences for ORC1 and -2 (GenBank™/EBI accession number AF323003, gene name SLC25A2) were amplified from human liver cDNA by PCR. Total RNA populations extracted from the fibroblasts of patients with HHH were reverse transcribed, and the coding sequences for ORC1 mutants were amplified by PCR with the aid of a GeneAmp® RNA PCR core kit (Applied Biosystems, Foster City, CA). The cDNA for ORC2 was amplified from the same six samples. The mutant forms of ORC1 corresponded to 7 alleles from 6 HHH patients (10, 11). Five of them were homozygous for the mutations G27R, R275Q, R179X, C861insG, and the other one was heterozygous for G190D and F188Δ. The recommended nomenclature for human gene mutations is that the adenosine of the first AUG codon of the mRNA from the
Expression Analysis by Real Time PCR—Total RNAs from human tissues (Invitrogen) were reverse transcribed with the GeneAmp® RNA PCR core kit (Applied Biosystems) with random hexamers as primers. The forward and reverse primers, based on the ORC1 and -2 cDNA sequences, were designed for real time PCRs with Primer Express (Applied Biosystems). The forward and reverse primers for ORC1 corresponded to nt 351–373 and 392–415, respectively, and those for ORC2 corresponded to nt 725–746 and 784–812, respectively. The ORC1 FAM/MGB- and the ORC2 VIC/MGB-Dark Quencher-labeled probes corresponded to nt 374–389 and 755–775 of the ORC1 and -2 DNA sequences, respectively. Real time PCRs were performed in a MicroAmp® optical 96-well plate using the automated ABI Prism 7000 sequence detector system (Applied Biosystems). The reaction mixture (50 μl) contained template (reverse transcribed first-stranded cDNA, 5 μl), 1× TaqMan Universal Master mix (Applied Biosystems), 200 nM probe for ORC1 or -2, and 900 nM each primer. To correct for differences in the amount of starting first-stranded cDNA, the human β-actin was amplified in parallel as a reference endogenous housekeeping gene. The relative quantification of the two isoforms was performed according to the comparative method (2\(^-\Delta\Delta Ct\)) (Ref. 13 and Applied Biosystems User Bulletin 2 P/N 43038559), with the heart ΔCt for ORC1 and the spleen ΔCt for ORC2 as the internal calibrator. 2\(^-\Delta\Delta Ct\) equals 2\(^-\Delta Ct\sample – \Delta Cteenzyme\), where ΔCt sample is Ct sample – Ct reference gene and Ct is the threshold cycle, i.e. the PCR cycle number at which emitted fluorescence exceeds 10× standard deviation of baseline emissions. 

**Bacterial Expression and Purification of ORC1, ORC2, and Mutated Forms of ORC1—**ORC1 and its mutated forms as well as ORC2 were overexpressed as inclusion bodies in the cytosol of *E. coli* as described before (12), except that the host cells were *E. coli* CO214(DE3) (14, 15). Control cultures with the empty vector were processed in parallel. Plasmids were isolated by centrifugation (258,000 × g for 1 h), and the 15 mg of protein, 100 μM ornithine (except where indicated otherwise), 10 mM HEPES, pH 7.2, carboxin (0.6 mg, Sigma), and water to a final volume of 700 μl of these compounds. The mixture was mixed thoroughly, and the mixture was recycled 13 times through the same Amberlite column (Bio-Rad). 

**Transport Assays—**External substrate was removed from proteoliposomes on columns of Sephadex G-75, which was pre-equilibrated with 50 mM Tris-HCl, pH 7.2. Transport at 25 °C was then started by adding 1\(^-\)H]ornithine, 1\(^-\)H]hylleline, 1\(^-\)H]arginine, 1\(^-\)H]cysteine, and 1\(^-\)H]citrulline (PerkinElmer Life Sciences) to substrate-loaded proteoliposomes (exchange) or to empty proteoliposomes (unidirectional transport). In both cases, transport was terminated by addition of 15 mM pyridoxal 5'-phosphate (the "inhibitor-stop" method) (16). In controls, the inhibitor was added at the beginning with the radiolabeled substrate. All transport measurements were carried out at the same internal and external pH value of 7.2. Finally, the external substrate was removed, and the radioactivity in the liposomes was measured (16). The experimental values were corrected by subtracting control values, and the transport activities were calculated by taking into account the efficiency of reconstitution (i.e. the yield of successfully reconstituted protein). The initial transport rate was calculated from the radioactivity taken up by proteoliposomes in 2 min (in the initial linear range of substrate uptake). Alternatively, the initial transport rate was calculated from the time course of isotope equilibration (16). The reconstituted proteins were assayed for other exchange activities by the inhibitor-stop method.

**RESULTS**

Identification of Human Isoform ORC2—By screening data bases with the sequence of ORC1, an intron-less gene interrupting the human protoncaderhin gene cluster (GenBank™, EBI accession number AF532005, Ref. 9) was found to encode a protein 301 amino acids long that is 87% identical in sequence to ORC1. This protein is isofrom ORC2.

Expression of the mRNAs for Human ORC1 and -2 in Various Tissues—The tissue distribution of mRNAs for isofroms ORC1 and -2 is summarized in Fig. 1. In all of these tissues, the ORC1 mRNA was expressed at higher levels than the ORC2 mRNA. The amount of the ORC1 mRNA in heart is about the same as that of the ORC2 mRNA in spleen, and as such this value served as an internal calibration in the relative quantification of the isofroms in various tissues. The ORC1 mRNA was expressed most strongly in liver, pancreas, testis, lung, and small intestine, and lower levels were detected in spleen, kidney, brain, and heart. The ORC2 mRNA was expressed in reasonable abundance in liver, testis, spleen, lung, pancreas, and small intestine and expressed poorly in other tissues. The ratio of ORC1 mRNA to ORC2 mRNA was 41 in brain, 93 in heart, 81 in kidney, 33 in liver, 65 in lung, 161 in pancreas, 38 in small intestine, 9.2 in spleen, and 18 in testis. However, because of the possibility of the operation of post-transcriptional mechanisms, these levels do not necessarily reflect the ratios of transport activities.

Bacterial Overexpression of ORC1 and -2—Both isofroms were expressed at high levels in *E. coli* CO214(DE3) (see Fig. 2, lanes 5 and 6) and about 70 mg of purified proteins were obtained per liter of culture. The proteins were not detected either in bacteria harvested immediately before induction of expression (lanes 1–3) or in control (with only vector) cells harvested after the induction (lane 4). Purified ORC1 and -2 (see Fig. 2, lanes 7 and 8) had apparent molecular masses of 31.5 and 31.0 kDa, respectively. The calculated values, including the initiator methionine, are 32714 and 32558 Da, respectively. All of the purified ORC1 mutant proteins gave single bands on SDS-PAGE. Their apparent molecular masses were 31.5 kDa for mutants G27R (Fig. 2, lane 9), G190D, R275Q, and F188A (data not shown), 26.0 kDa for mutant IV5S1+1g-α.
directional transport) of 10 mM internal ornithine are compared to ornithine measured in the presence (exchange) or absence (uni-directional transport) of 1 mM internal ornithine. They did not catalyze homo-exchanges for ADP, alanine, aspartate, ATP, carnitine, choline, citrate, glutamate, glutathione, malate, malonate, oxoglutarate, phosphate, proline, pyruvate, spermine, threonine, and valine. Control proteoliposomes made with boiled samples of ORC1 and -2 and with sarkosyl-solubilized material from bacterial cells either lacking expression vectors for ORC1 and -2 or harvested immediately before induction of expression had no [3H]ornithine-ornithine exchange activity.

The kinetics of uptake into proteoliposomes of 1 mM [3H]ornithine measured in the presence (exchange) or absence (uni-directional transport) of 10 mM internal ornithine are compared in Fig. 3. In both modes, isotopic equilibrium was approached exponentially in accord with transport by first-order kinetics. The ratio of maximal substrate uptake by exchange and by unidirectional transport was 9.0 for ORC1 and 11.3 for ORC2, agreeing with the value of 10 expected from intraliposomal concentrations at equilibrium of 1 and 10 mM for unidirectional transport and exchange, respectively. The addition of 10 mM unlabeled ornithine after incubation for 30 min (ORC1) or 120 min (ORC2) when radioactive uptake by the proteoliposomes had almost approached equilibrium caused an extensive efflux of radiolabeled ornithine from both ornithine-loaded and unloaded proteoliposomes (data not shown). This efflux shows that the [3H]ornithine taken up by unidirectional transport is released by exchange for externally added substrate. Therefore, ORC1 and -2 catalyze both the unidirectional transport of ornithine and ornithine-ornithine exchange, as reported also for the ornithine carrier from rat liver mitochondria and the recombinant yeast ortholog (1, 4, 7).

The substrate specificities of ORC1 and -2 were examined by measuring the uptake of [3H]ornithine into proteoliposomes pre-loaded with potential substrates (Fig. 4). With ORC1, the highest activities were observed in the presence of internal L-ornithine, L-lysine, L-arginine, and L-citrulline (see Fig. 4A). The D-isomers were much less effective. The activities detected in the presence of internal histidine, carnitine, cysteine, phenylalanine, and valine and (data not shown) alanine, γ-amino butyrate, citrate, glutamate, leucine, proline, serine, and threonine were virtually the same as the activity observed without internal substrate. Virtually no activities were detected with internal spermine or spermidine, suggesting that they inhibit ornithine transport by ORC1. In contrast, with internal malate or phosphate, [3H]ornithine uptake was greater than in the absence of internal substrate.

Isoform ORC2 behaved rather differently (see Fig. 4B), and high [3H]ornithine transport was observed with both L- and D-isomers of internal ornithine, lysine, arginine, and histidine. The rate of the unidirectional transport of ornithine by ORC2 was about 25% of the rate of ornithine-ornithine exchange. Furthermore, with internal citrulline the rate of [3H]ornithine uptake compared with that of ornithine uptake with no internal substrate was enhanced less with ORC2 than with ORC1. However, as observed with ORC1, the [3H]ornithine uptake by ORC2 was the same with no internal substrate as with internal carnitine, cysteine, phenylalanine, and valine and (data not shown) alanine, γ-amino butyrate, glutamate, leucine, proline, and threonine; there was very low activity with internal spermine and spermidine and significant activity with internal malate and phosphate.
As ORC1 and -2 did not catalyze either [32P]phosphate-phosphate or [14C]malate-malate homo-exchanges (see above), the effect of internal malate or phosphate on the uptake of [3H]ornithine was investigated further. As shown in Table I, [3H]ornithine uptake by ORC1 or -2 was enhanced not only with internal malate or phosphate but also with internal malonate, succinate, fumarate, aspartate, glutamate, and oxoglutarate into liposomes (data not shown), demonstrating that dicarboxylates and phosphate are not exchanged for basic amino acids. Furthermore, external phosphate, malate, and the other dicarboxylates neither induced efflux of internal [3H]ornithine nor stimulated uptake of [3H]ornithine. Only at high concentrations with a Ki higher than 10 mM did these compounds inhibit the carrier-mediated uptake of [3H]ornithine. Therefore, malate, phosphate, and other dicarboxylates may regulate the rate of ornithine uptake by reacting with ORC1 and -2 on the internal surface of the proteoliposomes.

The [3H]ornithine-ornithine exchange reactions catalyzed by ORC1 and -2 were inhibited strongly by pyridoxal 5’-phosphate (an inhibitor of several mitochondrial carriers) as well as by mercurials (mersalyl, p-chloromercuribenzenesulfonate, and mercuric chloride) and N-ethylmaleimide, which are powerful inhibitors of the ornithine carrier from rat liver (1). Both isoforms were inhibited strongly by spermine (Fig. 5), whereas carboxyatractysolide, bongkrekate, α-cyano-4-hydroxycinnamate, and brom cresol purple (inhibitors of other mitochondrial carriers) had little or no effect.

**Kinetic Characteristics**—The kinetic constants of ORC1 and -2 were determined from the initial transport rate of homo-exchanges at various external labeled substrate concentrations in the presence of a constant saturating internal substrate concentration of 20 mM. With both isoforms, linear functions were obtained in double reciprocal plots. For ORC1, the specific activities (Vmax) for homo-exchanges for ornithine, lysine, arginine, and citrulline were all about 3.0 ± 0.4 mmol/min/g of protein, and the transport affinities (Km) were 0.22 ± 0.02, 0.80 ± 0.06, 1.58 ± 0.18, and 2.52 ± 0.30 mM, respectively (in at least four experiments for each homo-exchange). For ORC2, the transport affinities were 0.40 ± 0.06, 0.32 ± 0.05, 0.71 ± 0.09, and 1.28 ± 0.14 mM, respectively, for L-isomers of ornithine, lysine, arginine, and histidine (in at least four experiments for each homo-exchange), and the Vmax values (1.2 ± 0.2 mmol/min/g of protein) were again independent of the substrate. Citrulline, lysine, spermine, and spermidine inhibited competitively [3H]ornithine uptake by both isoforms by increasing the apparent Km without changing the Vmax of ornithine uptake (data not shown). For ORC2, the inhibition constants (Ki) of citrulline, lysine, spermine, and spermidine were 8.1 ± 1.2, 0.37 ± 0.04, 0.30 ± 0.03, and 0.48 ± 0.06 mM, respectively (in at least three experiments for each inhibitor). For ORC1, the Ki values of citrulline, lysine, spermine, and spermidine were 0.09, 0.4, and 1.28 mM, respectively. 

![Image](81x348 to 283x737)

**Fig. 4.** Dependence of the transport properties of ORC1 and -2 on internal substrate. Proteoliposomes reconstituted with ORC1 (A) or ORC2 (B) were preloaded internally with various substrates (concentration of 20 mM). Transport was started by addition of 0.2 mM [3H]ornithine and was terminated after 2 min. Similar results were obtained in four different experiments for each carrier investigated.

| Internal substrate | [3H]Ornithine transport |
|--------------------|-------------------------|
|                    | ORC1 | ORC2 |
| None               | 117  | 150  |
| Malate             | 295  | 288  |
| Phosphate          | 289  | 284  |
| Malonate           | 222  | 199  |
| Succinate          | 146  | 188  |
| Fumarate           | 221  | 195  |
| Aspartate          | 230  | 207  |
| Glutamate          | 242  | 246  |
| 2-Oxoglutarate     | 213  | 169  |
| Asparagine         | 124  | 137  |
| Glutamine          | 117  | 145  |
| Acetocetate        | 110  | 153  |
Isoforms ORC1 and -2 have many transport properties in common. They both transport ornithine, lysine, arginine, and citrulline by exchange and unidirectionally, and they are inactivated by the same inhibitors. However, they also differ in a number of respects. First, ORC2 has a broader substrate specificity than ORC1. It transports L- and D-histidine, L-homoarginine, and D-isomers of ornithine, lysine, and arginine with the same efficiency as L-isomers. Second, the \( K_m \) values of ORC2 for ornithine and citrulline are higher. Third, ORC2 is three times less active than ORC1.

The transport properties of rat liver ORC (1, 2, 5) are more similar to human ORC1 than to ORC2. Neither the native rat ORC nor the recombinant human ORC1 transports histidine; they both prefer to transport L-isomers of other amino acids, and their transport affinities for ornithine, lysine, arginine, and citrulline are very similar. Thus, as in human liver, ORC1 is probably the predominantly expressed isoform in rat liver.

Because ORC1 transports citrulline better than ORC2 and because it is more abundant than ORC2 in liver, ORC1 is likely to carry out the important function of exchanging cytosolic ornithine and mitochondrial citrulline. Thus, it probably provides an essential link in the urea cycle between enzyme activities in the cytosol and others in the mitochondrial matrix. The lower activity of ORC1 in patients with HHH syndrome supports this interpretation. Moreover, the transport of lysine by ORC1 provides a means for inhibition of urea synthesis by lysine observed in isolated hepatocytes (18). The phenotype of HHH patients is generally milder than those associated with defects in any urea cycle enzyme. In HHH patients, inactive ORC1 is found alongside fully active ORC2 in liver mitochondria and can compensate partially for ORC1. However, because it has a lower affinity for ornithine and citrulline and is expressed at a lower level, it cannot replace completely the function of ORC1 in the urea cycle. Since in mouse the second gene involved, termed ORC2, is probably the predominantly expressed isoform in rat liver.
mitochondrial protein synthesis, and import of ornithine is needed for the degradation of excess arginine. When the dietary content of arginine is high, the production of ornithine exceeds the requirement for citrulline formation. This excess is removed by ornithine aminotransferase (19, 20) expressed in the mitochondrial matrix in response to the level of protein in the diet (21–23). In isolated rat liver mitochondria, ornithine aminotransferase catabolizes ornithine at a rate that is limited by transport of ornithine into the matrix (20). The hepatic enzyme is found in pericentral hepatocytes that contain glutamine synthetase and not in the periporal hepatocytes containing the urea cycle enzymes (24). Therefore, it seems possible that the ornithine-citrulline exchange activity of ORC1 and -2 occurs in mitochondria of periporal hepatocytes and that ornithine–H⁺ exchange is performed by the same transporters in mitochondria of the pericentral hepatocytes. Another possibility is that ORC2 is located mainly in mitochondria of pericentral hepatocytes and that ORC1 is in mitochondria of periporal hepatocytes.

The efflux of ornithine from mitochondria is required for polyamine biosynthesis from ornithine in the cytosol. When the diet content of arginine is low and/or in the tissues where the activity of arginase is negligible (25), ornithine made from glutamine is transported into mitochondria reducing the urea cycle enzymes (24). Therefore, it seems possible that the ornithine-citrulline exchange activity of ORC1 and -2 occurs in mitochondria of periporal hepatocytes and that ornithine–H⁺ exchange is performed by the same transporters in mitochondria of the pericentral hepatocytes. Another possibility is that ORC2 is located mainly in mitochondria of pericentral hepatocytes and that ORC1 is in mitochondria of periporal hepatocytes.

Another important observation is that the activities of ORC1 and -2 are stimulated by dicarboxylates, phosphate, and malate in various tissues at different levels, and both isoforms are inhibited by spermine and spermidine. Thus, polyamines in the cytosol could control their own synthesis by reducing the amount of ornithine exported from the mitochondria via the ornithine carrier.

If ORC1 and -2 are reconstituted into liposomes with the same orientation as in the mitochondria, malate could stimulate ureogenesis directly by activating ORC1 and -2 on the matrix side of the inner mitochondrial membrane. Because the intramitochondrial concentration of phosphate is high and the affinity of the ornithine carrier for dicarboxylates other than malate is low, the physiological effect of these dicarboxylates on the activity of the ORC may be minor.

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