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The mitochondrial ornithine transporter: bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms*#

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#Dedicated to the memory of Professor Eraldo Antonini.

Abbreviations: HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; nt, nucleotides; ORC, ornithine carrier protein; PCR, polymerase chain reaction; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

SUMMARY

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. They 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 preponderant form. The highest levels of expression of both isoforms are in liver. Five mutant forms of ORC1 associated with the human disease hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) were also made. The mutations abolish the transport properties of the protein. In patients with HHH, 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) (1-5). The reconstituted purified protein is highly active in ornithine/citrulline exchange and is somewhat less active in unidirectional transport of ornithine. It tranports lysine and arginine also, but not 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 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 catabolism of excess arginine and the biosynthesis of polyamines (4).

In *Saccharomyces cerevisiae*, the ornithine carrier (ORC) is encoded by ARG11/ORT1, and its identity was established by overexpression in bacteria, reconstitution and study of its transport properties (7). Subsequently, ORNT, 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, ORNT2, that in man encodes a second isoform, ORC2 (9). The isoforms are expressed differently in various human tissues. Both were

overexpressed in *E. coli,* and 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 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 (accession number AF112968, 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 ORC2 (accession number AF332005, gene name SLC25A2) were amplified from human liver cDNA by PCR. Total RNA populations extracted from the fibroblasts of patients with HHH were reversed transcribed, and the coding sequences for ORC1 mutants were amplified by PCR with the aid of a Gene Amp RNA PCR Core kit (Applied Biosystems, Foster City, CA, U. S. A.). 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 IVS5+1g→a, and the other one was heterozygous for G190D and F188Δ. The recommended nomenclature for human gene mutations is that the A of the first AUG codon of the mRNA from the SLC25A15 gene (GENBANK number NM_014252) is taken as nucleotide 1, and the corresponding methionine as residue 1 of the protein. The oligonucleotide primers

corresponded to the extremities of the coding sequences of ORC1 and ORC2 with additional NdeI and HindIII sites. The amplified products were cloned into the pRUN expression vector which is derived from pKN172 (12), and the constructs were transformed into *E. coli* TOP 10 cells (Invitrogen, Carlsbad, CA, U. S. A). Transformants were selected on 2xTY plates containing ampicillin (100 μg/ml) and were screened by direct colony PCR and restriction digestion of plasmids.

Expression analysis by real-time PCR— Total RNAs from human tissues (Invitrogen) were reverse-transcribed with the Gene Amp RNA PCR Core kit (Applied Biosystems) with random hexamers as primers. Primers and probes, based on the ORC1 and ORC2 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 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 the ORC2 cDNA 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), 1X TaqMan Universal Master Mix (Applied Biosystems), 200 nM probe for ORC1 or ORC2, and 900 nM of each primer. To correct for differences in the amount of starting first-stranded cDNAs, the human β-actin gene was amplified in parallel as a reference endogenous housekeeping gene. The relative quantification of the two isoforms was performed according to the comparative method ($2^{-\Delta\Delta Ct}$) ((13) and Applied Biosystems User Bulletin No.2 P/N 4303859), with the heart ΔCt for ORC1 and the spleen ΔCt for ORC2 as internal calibrator. $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ calibrator})}$, 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 the 10 times standard deviation of baseline emissions.

Bacterial expression and purification of ORC1, ORC2 and mutated forms of ORC1 - ORC1 and its mutated forms, and 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. Inclusion bodies were purified on a sucrose density gradient (12), washed at 4°C, first with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 6.5), then twice with a buffer containing Triton X-114 (3%, w/v), 1 mM EDTA and 10 mM HEPES pH 7.2, and finally with TE buffer. The proteins were solubilized in 1.8% sarkosyl (w/v). Small residues were removed by centrifugation (258000 x g, 1 h).

Reconstitution into liposomes - Recombinant proteins in sarkosyl were reconstituted into liposomes in the presence or absence of substrates (16). The reconstitution mixture contained solubilized proteins (150 µl; 1-1.2 µg of protein), 10% Triton X-114 (70 µl), 10% phospholipids as sonicated liposomes (80 µl), 20 mM ornithine (except where indicated otherwise), 10 mM HEPES, pH 7.2, cardiolipin (0.8 mg; Sigma, St. Louis, MO, U. S. A.), and water to a final volume of 700 µl. These components were mixed thoroughly, and the mixture was recycled 13 times through the same Amberlite column (Bio-Rad, Philadelphia, PA, U. S. A.).

Transport assays — External substrate was removed from proteoliposomes on columns of Sephadex G-75, pre-equilibrated with 50 mM sucrose and 10 mM HEPES, pH 7.2. Transport at 25°C was started by adding L-[^{3}H] ornithine, L-[^{3}H]lysine, L-[^{3}H]arginine, L-[^{14}C]histidine or L-[^{14}C]citrulline (Perkin Elmer Life Sciences, Boston, MA, U. S. A.) 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 "inibitor-stop" method (16)). In controls, the inhibitor was added at the beginning with the radioactive 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 incorporated 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.

Other methods - Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue dye. N-terminal sequencing was carried out as described before (17). The amount of ORC1 and ORC2 was estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard (17). The amount of protein incorporated into liposomes was measured as described previously (17). It varied between 24-32% of the protein added to the reconstitution mixture.

RESULTS

Identification of human isoform ORC2 — By screening data bases with the sequence of ORC1, an intron-less gene interrupting the human protocadherin gene cluster (accession no. AF332005; (9)) was found to encode a protein 301 amino acids long that is 87% identical in sequence to ORC1. This protein is isoform ORC2.

Expression of the mRNAs for human ORC1 and ORC2 in various tissues — The tissue distribution of mRNAs for isoforms ORC1 and ORC2 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 so this value served as an internal calibration in the relative quantification of the isoforms 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 poorly in other tissues. The ratio of ORC1 mRNA : 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 ORC2 — Both isoforms were expressed at high levels in *E. coli* C0214(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 ORC2 (see Fig. 2, lanes 7 and 8) had apparent molecular masses of 31.5 kDa 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 gels. Their apparent molecular masses were 31.5 kDa for mutants G27R (Fig. 2, lane 9), G190D, R275Q and F188Δ (not shown), 26.0 kDa for mutant IVS5 + 1g→a (Fig. 2, lane 10), 30.5 kDa for mutant c861insG

(Fig. 2, lane 11) and 21.5 kDa for mutant R179X (Fig. 2, lane 12). The identities of all proteins were confirmed by N-terminal sequencing.

Transport properties of recombinant ORC1 and ORC2 — In homo-exchange assays (same substrate inside and outside at external and internal concentrations of 1 and 10 mM, respectively), reconstituted ORC1 and ORC2 both catalyzed a [^{3}H]ornithine/ornithine exchange that was inhibitable by pyridoxal 5'-phosphate. 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 ORC2, and with sarkosyl-solubilized material from bacterial cells either lacking expression vectors for ORC1 and ORC2, or harvested immediately before induction of expression, had no [^{3}H]ornithine/ornithine exchange activity.

The kinetics of uptake into proteoliposomes of 1 mM [^{3}H]ornithine measured in the presence (exchange) or absence (unidirectional 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 [^{3}H]ornithine taken up by unidirectional transport is released by exchange for externally

added substrate. Therefore, ORC1 and ORC2 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 ORC2 were examined by measuring the uptake of [^{3}H]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, valine and (not shown), alanine, γ-aminobutyrate, citrate, glutamine, 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 [^{3}H]ornithine uptake was greater than in the absence of internal substrate.

Isoform ORC2 behaved rather differently (see Fig. 4B), and high [^{3}H]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 [^{3}H]ornithine uptake, compared with that of ornithine uptake with no internal substrate, was less enhanced with ORC2 than with ORC1. However, as observed with ORC1, the [^{3}H]ornithine uptake by ORC2 was the same with no internal substrate as with internal carnitine, cysteine, phenylalanine, valine and (not shown), alanine, γ-aminobutyrate, glutamine, 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 ORC2 did not catalyze either [^{32}P]phosphate/phosphate or [^{14}C]malate/malate homo-exchanges (see above), the effect of internal malate or phosphate on the uptake of [^{3}H]ornithine was investigated further. As shown in Table I, [^{3}H]ornithine uptake by ORC1 or ORC2 was enhanced, not only with internal malate or phosphate, but also with internal malonate, succinate, fumarate, aspartate, glutamate and oxoglutarate in comparison with proteoliposomes with no internal substrate. In contrast, with asparagine, glutamine, acetoacetate and (not shown) propionate, butyrate, γ-aminobutyrate and 2-oxoisocaproate, the transport of [^{3}H]ornithine by both isoforms was virtually the same as in the absence of internal substrate. Results similar to those illustrated in Table I were found with [^{3}H]lysine, and, for ORC2, with [^{14}C]histidine, instead of [^{3}H]ornithine. The ORC-mediated, increased transport of basic amino acids by phosphate, malate and other dicarboxylates was concentration dependent. Half-maximal stimulation of [^{3}H]ornithine uptake required about 2.2 mM malate, 3.9 mM phosphate, and a concentration higher than 7.5 mM of other dicarboxylates. In the presence of internal ornithine, lysine or arginine both isoforms did not catalyze the uptake of labeled malate, phosphate, malonate, fumarate, aspartate, glutamate and oxoglutarate into liposomes (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 [^{3}H]ornithine nor stimulated uptake of [^{3}H]ornithine. Only at high concentrations, with a Ki higher than 10 mM, did these compounds inhibit the carrier-mediated uptake of [^{3}H]ornithine. Therefore, malate, phosphate and other dicarboxylates may regulate the rate of ornithine uptake by reacting with ORC1 and ORC2 on the internal surface of the proteoliposomes.

The [^{3}H]ornithine/ornithine exchange reactions catalyzed by ORC1 and ORC2 were inhibited strongly by pyridoxal 5'-phosphate (an inhibitor of several mitochondrial carriers) as well as by mercurials (mersalyl, p-chloromercurybenzene sulfonate and mercuric chloride) and by N-ethylmaleimide, which are powerful inhibitors of the ornithine carrier from rat liver (1). Both isoforms were inhibited strongly by spermine (Fig. 5), whereas carboxyatractyloside, bongkrekate, α-cyano-4-hydroxycinnamate and bromocresolpurple (inhibitors of other mitochondrial carriers) had little or no effect.

Kinetic characteristics — The kinetic constants of ORC1 and ORC2 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 protein, and the transport affinities (Km) were 0.22 ± 0.02 mM, 0.80 ± 0.06 mM, 1.58 ± 0.18 mM, and 2.52 ± 0.30 mM, respectively (in at least 4 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, respectively for L-isomers of ornithine, lysine, arginine and histidine (in at least 4 experiments for each homo-exchange), and the Vmax values (1.2 ± 0.2 mmol / min / g protein) were again independent of the substrate. Citrulline, lysine, spermine and spermidine inhibited competitively [^{3}H]ornithine uptake by both isoforms, by increasing the apparent Km without changing the Vmax of ornithine uptake (not shown). For ORC2, the inhibition constants (Ki) of citrulline, lysine, spermine and spermidine were 8.1 ± 1.2 mM, 0.37 ± 0.04 mM, 0.30 ±

0.03 mM and 0.48 ± 0.06 mM, respectively (in at least 3 experiments for each inhibitor). For ORC1, the Ki values of citrulline, lysine, spermine and spermidine were 2.2 ± 0.3 mM, 0.87 ± 0.07 mM, 0.98 ± 0.15 mM and 1.4 ± 0.2 mM, respectively (in at least 3 experiments for each inhibitor).

Transport properties of mutant forms of ORC1 — The seven mutant forms of ORC1 were expressed in *E. coli* and reconstituted into liposomes just as well as the wild-type protein. Five of them, G27R, R275Q, R179X (residues 1-178 of ORC1), C861insG (residues 1-290), and IVS5+1g→a (ORC1 lacking transmembrane segments 5 and 6), were incapable of catalyzing homo-exchanges of ornithine, arginine, lysine and citrulline (Fig. 6). Even after 30 min incubation in the presence of external substrate concentrations 15 times higher than the Km, no activity was detected (not shown). It is likely that their inability to transport ornithine and citrulline leads to the HHH syndrome. The other two mutant forms of ORC1, i.e. G190D and F188Δ, mantained a residual transport activity of approximately 35 and 10%, respectively. They occurred in different alleles of a patient who did not show a milder phenotype compared to the others. He presented with neonatal onset hyperammonemia and developed spastic paraparesis in early adulhood (10). However, the lack of a clear genotype-phenotype correlation is often lacking in human diseases. In all six patients, the sequence of ORC2 was unaltered, and, from semi-quantitative RT-PCR experiments, it appeared that expression levels in cultured fibroblasts do not differ significantly from the levels found in control fibroblasts.

DISCUSSION

Isoforms ORC1 and ORC2 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 Km values of ORC2 for lysine and arginine are lower than for ORC1, and those 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 ORC1 than to ORC2. Neither the native rat ORC nor the recombinant 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 it is expressed at a lower level, it cannot replace completely the function of ORC1 in the urea cycle. Since in mouse the ORNT2 is a pseudogene (for the presence of a single nt deletion near the 5'-end causing a frame shift) (9), it would be interesting to delete the

murine ORNT1 and compare the resulting phenotype with that of a urea cycle enzyme knock-out mice.

Isoform ORC1 and especially isoform ORC2 are likely to play a variety of important roles in cell metabolism. The import of lysine, arginine and histidine into mitochondria is required for 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 periportal hepatocytes containing the urea cycle enzymes (24). Therefore, it seems possible that the ornithine/citrulline exchange activity of ORC1 and ORC2 occurs in mitochondria of periportal 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 periportal 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 glutamate inside mitochondria (21-23) has to be exported for polyamine biosynthesis. In intestinal mitochondria ornithine is made from glutamine and so could provide extra urea cycle intermediates for the liver (26). ORC1 and ORC2 are expressed 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.

Another important observation is that the activities of ORC1 and ORC2 are stimulated by dicarboxylates, phosphate and malate from the inside of the proteoliposomes, but as none of these compounds is transported by ORC1 and ORC2, the simplest explanation of this regulation is that malate and phosphate interact at a site distinct from the substrate-binding site. This site can bind C3-C5 dicarboxylates, but not asparagine, glutamine or monocarboxylates. Malate increases urea synthesis in isolated hepatocytes first, by transporting into mitochondria reducing equivalents and the carbon skeleton of fumarate produced in the cytosol by argininosuccinate lyase, and second, by producing aspartate inside the mitochondria via the malate dehydrogenase and aspartate aminotransferase (27). As aspartate is a substrate of argininosuccinate synthetase, it is essential for the functioning of the urea cycle. If ORC1 and ORC2 are reconstituted into liposomes with the same orientation as in the mitochondria, malate could stimulate ureogenesis directly by activating ORC1 and ORC2 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, their physiological effect on the activity of the ORC may be minor.

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FIGURE LEGENDS

FIG. 1. **Expression of human ORC1 and ORC2 in various tissues.** Real-time PCR experiments were conducted on cDNAs prepared by reverse-transcription of total RNAs from various human tissues, using specific primers and probes based on human ORC1 and ORC2. The relative quantification of human ORC1 (black bars) and ORC2 (grey bars) was performed by the comparative method ($2^{-\Delta\Delta Ct}$). Human β-actin was employed as reference.

FIG. 2. **Overexpression in E. coli and purification of ORC1, ORC2 and ORC1 mutants.** Proteins were separated by SDS-PAGE and stained with Coomassie Blue dye. Lanes M, markers (ovotransferrin, bovine serum albumin, ovoalbumin, carbonic anhydrase, myoglobin, and cytochrome c); lanes 1-6, *E. coli* C0214(DE3) containing the expression vector, without (lanes 1 and 4) and with the coding sequence of ORC1 (lanes 2 and 5) or ORC2 (lanes 3 and 6). Samples were taken at the time of induction (lanes 1-3) and 5 h later (lanes 4-6). The same number of bacteria was analyzed in each sample. Lanes 7 and 8, ORC1 and ORC2 purified from bacteria in lanes 5 and 6, respectively. Lanes 9-12, recombinant ORC1 mutants (G27R, IVS5+1g→a, c861insG and R179X, respectively) purified as the wild-type protein reported in lane 7.

FIG. 3. **Kinetics of [^{3}H]ornithine unidirectional transport and [^{3}H]ornithine/ornithine exchange by ORC1 and ORC2.** ORC1 was reconstituted into proteoliposomes (panel A) or ORC2 (panel B). 1 mM [^{3}H]ornithine was added to proteoliposomes containing 10 mM ornithine (exchange, ■) or 20 mM sucrose and no substrate (unidirectional transport, ●).

FIG. 4. **Dependence of the transport properties of ORC1 and ORC2 on internal substrate.** Proteoliposomes reconstituted with ORC1 (A) or

ORC2 (B) were preloaded internally with various substrates (concentration, 20 mM). Transport was started by addition of 0.2 mM (A) and 0.4 mM (B) [^{3}H]ornithine, and terminated after 2 min. Similar results were obtained in four different experiments for each carrier investigated.

FIG. 5. **Effect of inhibitors on the [^{3}H]ornithine/ornithine exchange by ORC1 and ORC2.** Proteoliposomes were preloaded internally with 20 mM ornithine and transport was started by adding 0.2 mM or 0.4 mM [^{3}H]ornithine to proteoliposomes reconstituted with ORC1 (black bars) or ORC2 (grey bars), respectively. The incubation time was 2 min. Thiol reagents were added 2 min before the labeled substrate; the other inhibitors together with labeled substrate. The final concentrations of the inhibitors were 0.1 mM (MER, mersalyl; p-HMBS, p-hydroxymercuribenzene sulfonate; BrCP, bromocresol purple; CCN α-cyanocinnamate), 10 mM (PLP, pyridoxal 5'-phosphate; BAT, bathophenanthroline), 1 mM (NEM, N-ethylmaleimide), 10 μM ($HgCl_2$; CAT, carboxyatractyloside; BKA bongkrekic acid), and 5 mM spermine. The extents of inhibition (%) from a representative experiment for each carrier are reported. Similar results were obtained in at least three independent experiments.

FIG. 6. **Transport assays of wild-type and mutant ORC1.** Recombinant, reconstituted wild-type and mutant ORC1 were assayed for their ability to catalyze the homo-exchanges [^{3}H]ornithine/ornithine (black bars), [^{3}H]arginine/arginine (white bars), [^{3}H]lysine/lysine (grey bars) and [^{14}C]citrulline/citrulline (dotted bars). Proteoliposomes were preloaded internally with 20 mM substrate and transport was started by adding 0.2 mM [^{3}H]ornithine, 1.5 mM [^{3}H]arginine, 0.8 mM [^{3}H]lysine and 2.5 mM [^{14}C]citrulline, respectively. The reaction time was 2 min. Similar results were obtained in three different experiments.

TABLE I

Stimulation of reconstituted ORC1 and ORC2 by phosphate, malate and other dicarboxylates.

Proteoliposomes were pre-loaded internally with various substrates (concentration, 20 mM). Transport was started by adding 1 mM [^{3}H]ornithine to proteoliposomes reconstituted with ORC1 or ORC2, and terminated after 2 min. Similar results were obtained in at least three independent experiments for each carrier investigated.

	[^{3}H]Ornithine transport (μmol / min / g protein)	
Internal substrate	ORC1	ORC2
none	117	150
malate	295	268
phosphate	269	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
acetoacetate	110	153

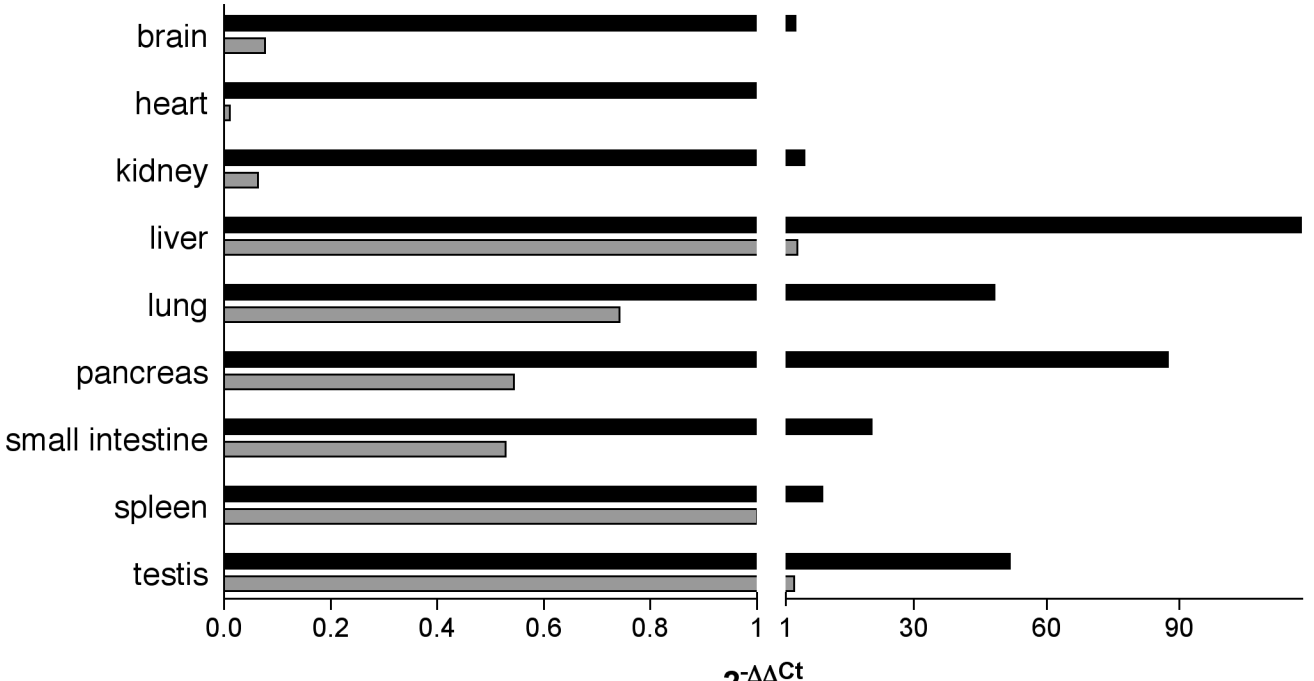

brain
heart
kidney
liver
lung
pancreas
small intestine
spleen
testis
0.0
0.2
0.4
0.6
0.8
1
1
30
60
90
2-ΔΔCt

Figure 1

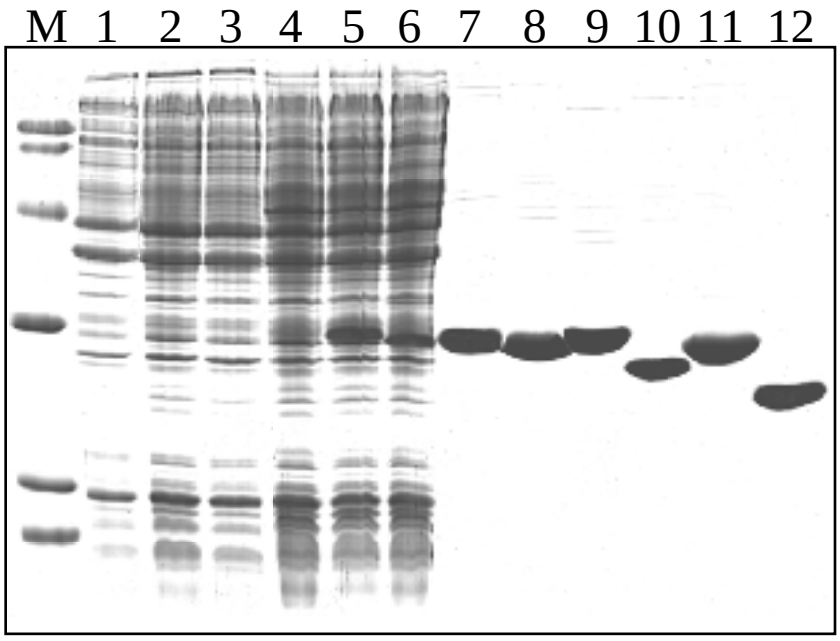
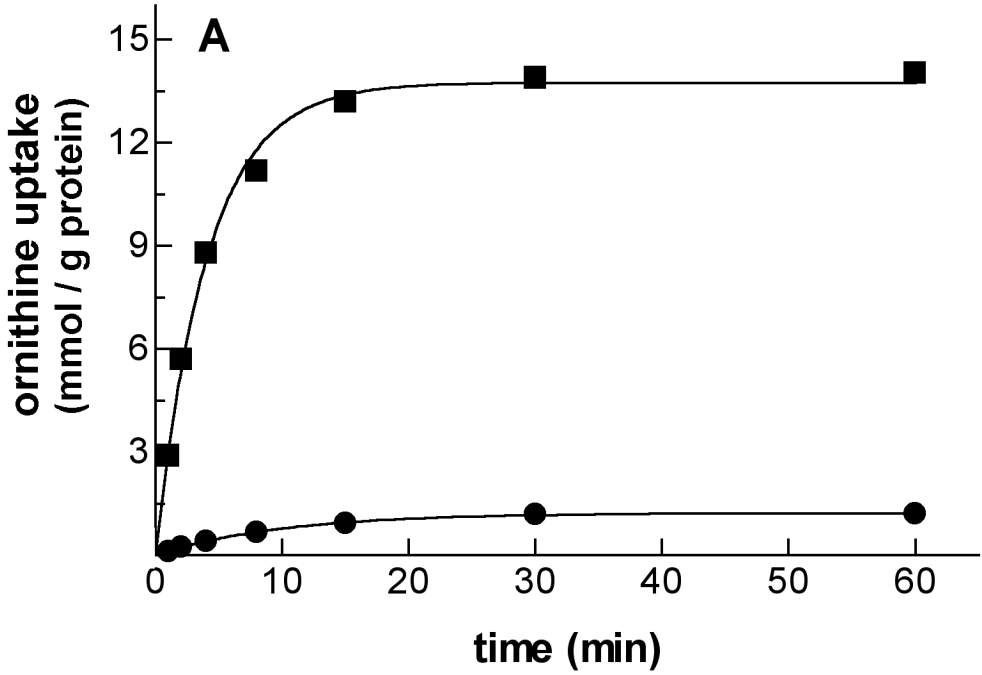


Figure 2


A
ornithine uptake
(mmol / g protein)
15
12
9
6
3
0
10
20
30
40
50
60
time (min)

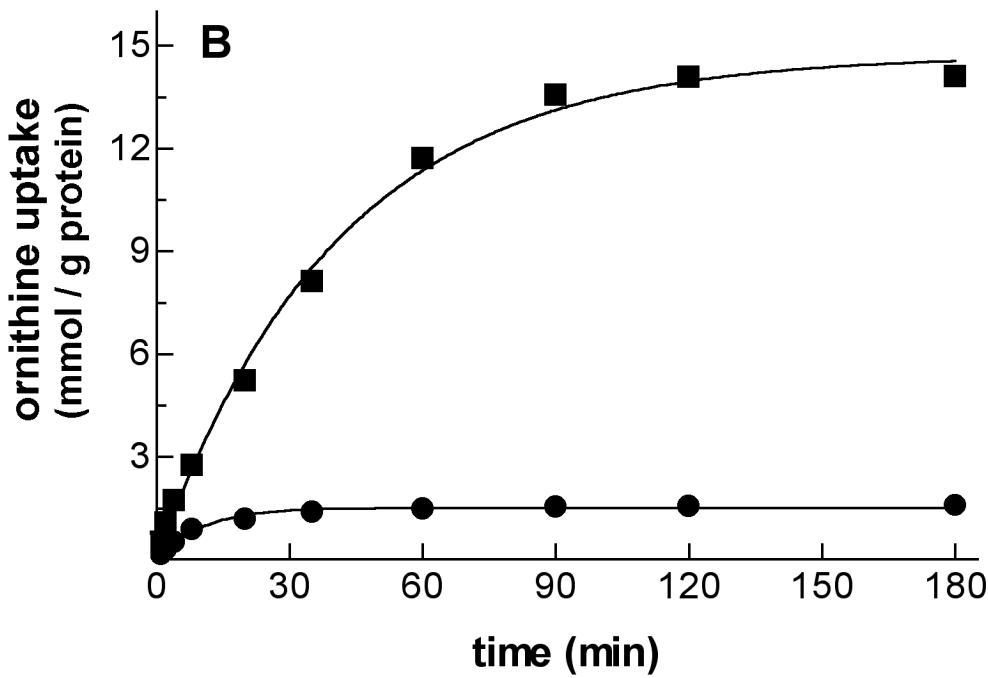

B
ornithine uptake
(mmol / g protein)
15
12
9
6
3
0
30
60
90
120
150
180
time (min)

Figure 3

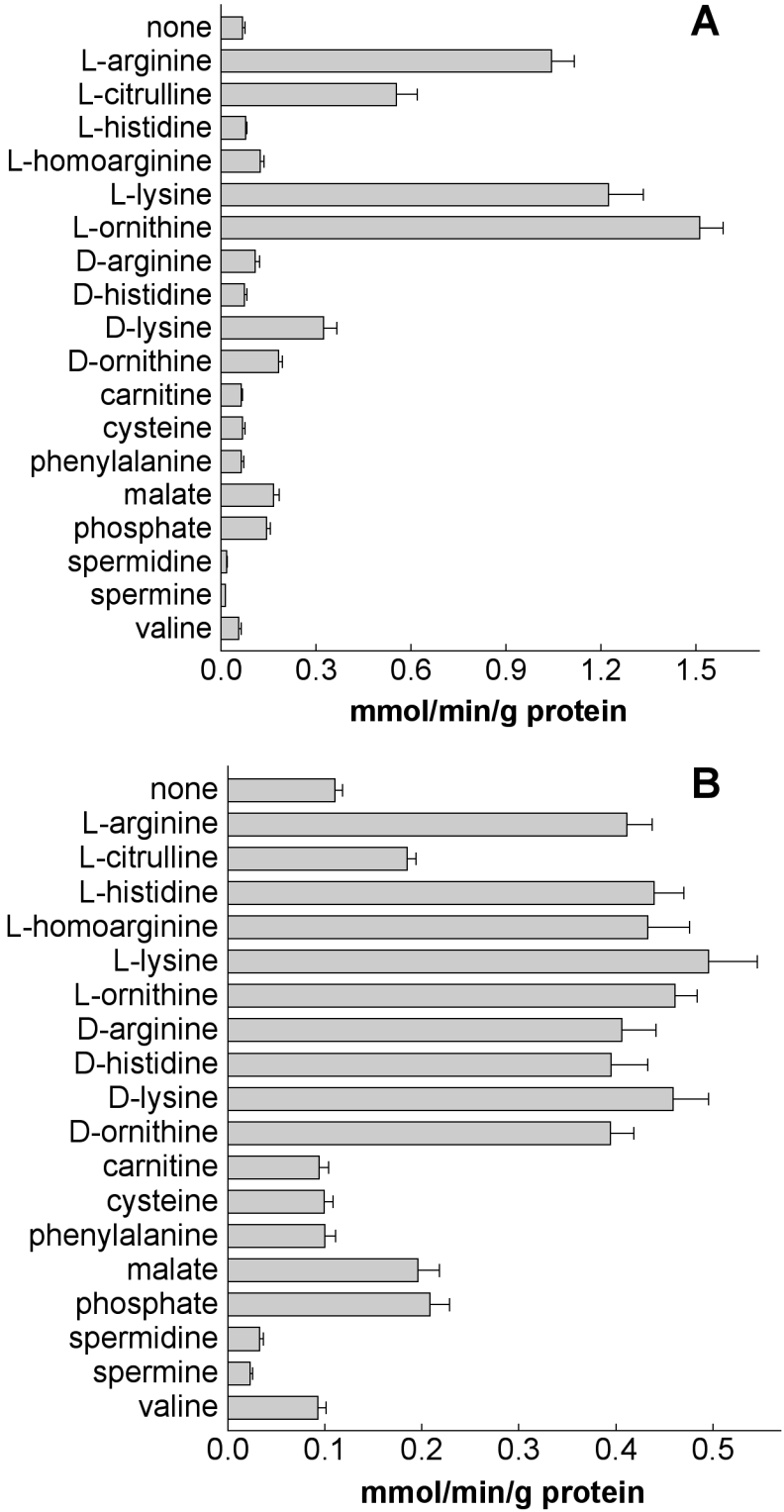

A
none
L-arginine
L-citrulline
L-histidine
L-homoarginine
L-lysine
L-ornithine
D-arginine
D-histidine
D-lysine
D-ornithine
carnitine
cysteine
phenylalanine
malate
phosphate
spermidine
spermine
valine
0.0
0.3
0.6
0.9
1.2
1.5
mmol/min/g protein
B
none
L-arginine
L-citrulline
L-histidine
L-homoarginine
L-lysine
L-ornithine
D-arginine
D-histidine
D-lysine
D-ornithine
carnitine
cysteine
phenylalanine
malate
phosphate
spermidine
spermine
valine
0.0
0.1
0.2
0.3
0.4
0.5
mmol/min/g protein

Figure 4

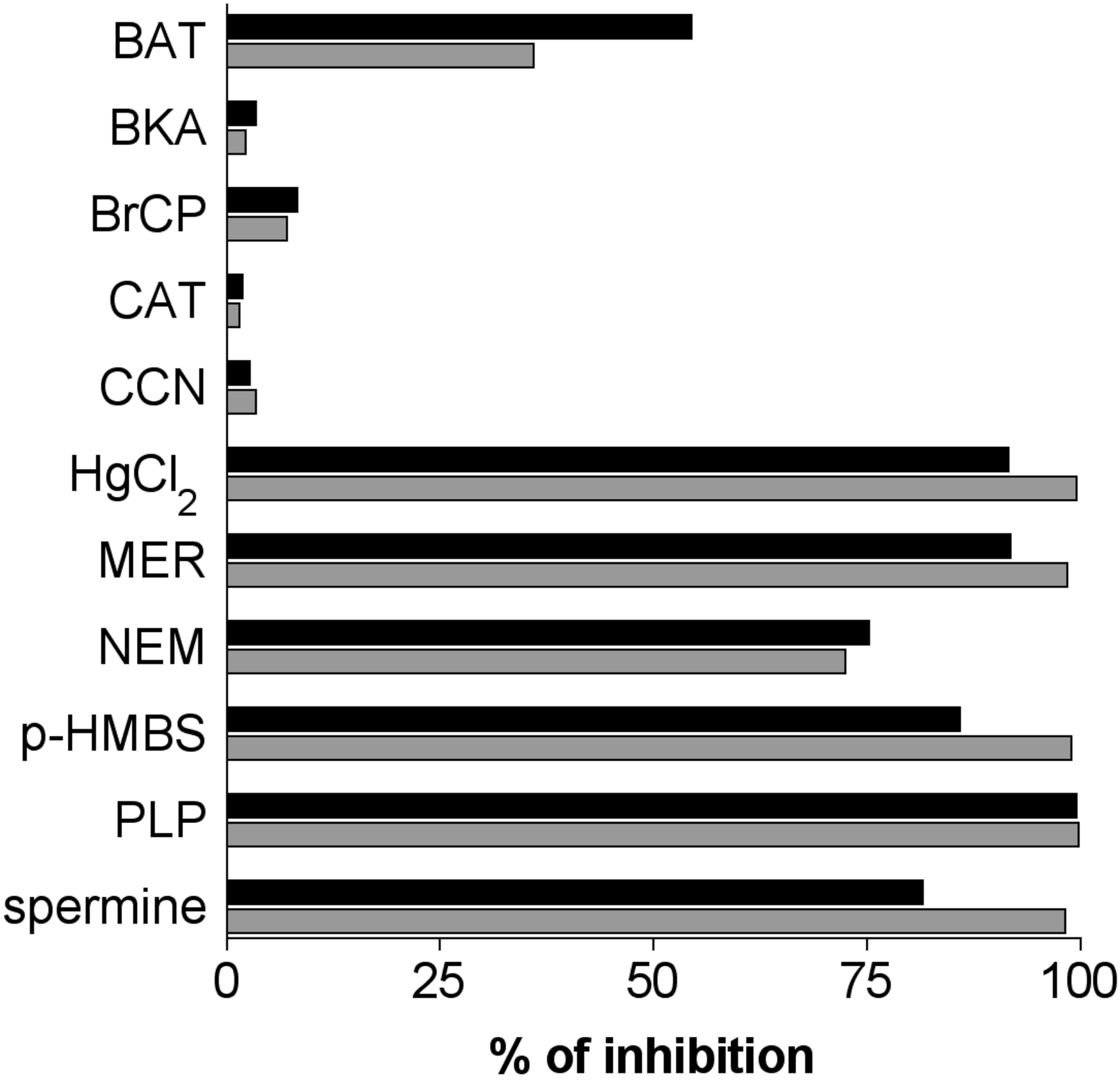

BAT
BKA
BrCP
CAT
CCN
HgCl2
MER
NEM
p-HMBS
PLP
spermine
0
25
50
75
100
% of inhibition

Figure 5

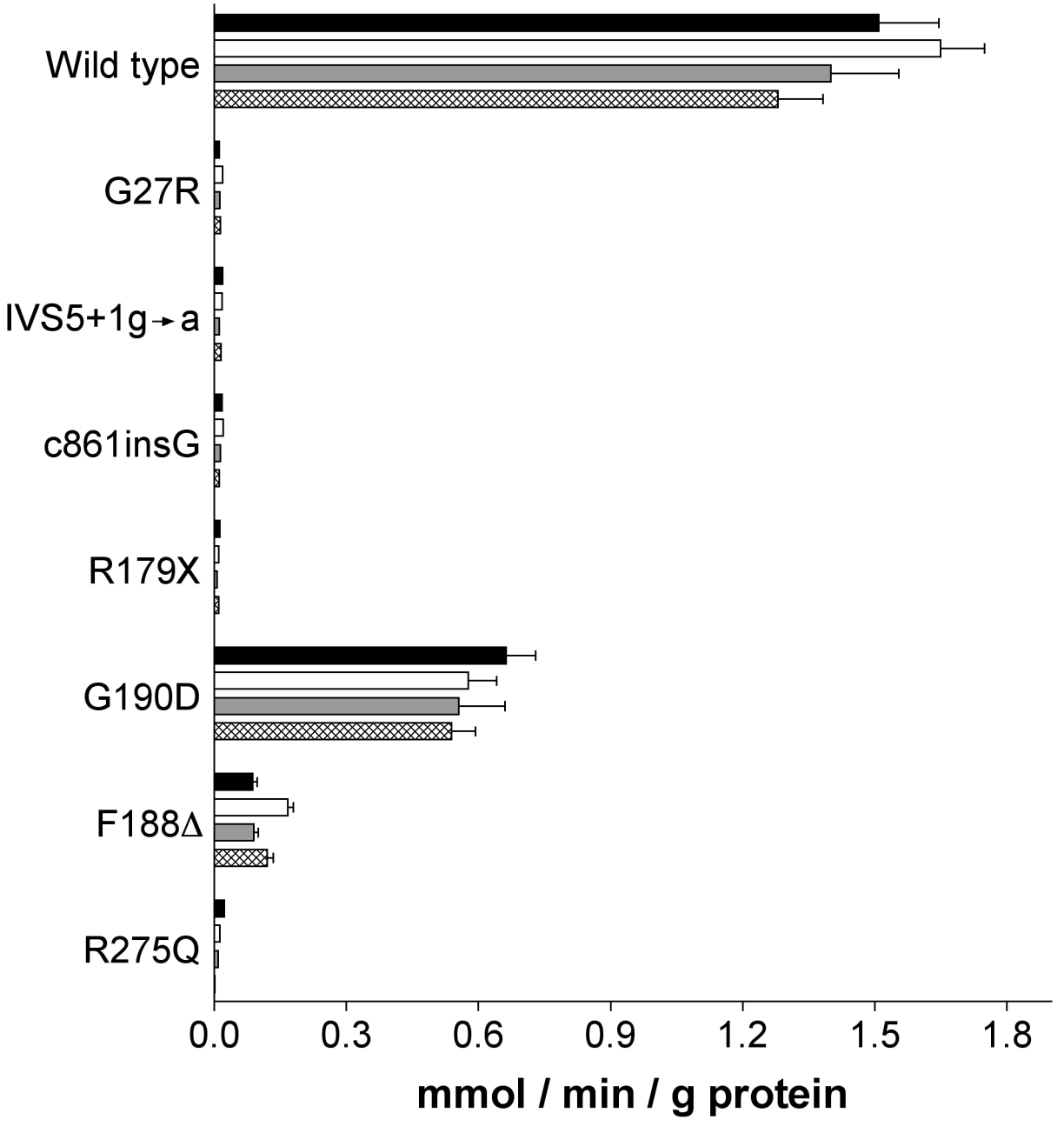

Wild type
G27R
IVS5+1g→a
c861insG
R179X
G190D
F188Δ
R275Q
0.0
0.3
0.6
0.9
1.2
1.5
1.8
mmol / min / g protein

Figure 6

The mitochondrial ornithine transporter: bacterial expression,reconstitution, functional characterization, and tissuedistribution of two human isoforms

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