THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 11, pp. 7925–7934, March 9, 2012

Substrate Specificity of the Two Mitochondrial Ornithine Carriers Can Be Swapped by Single Mutation in Substrate Binding Site*[S]

Received for publication, November 16, 2011, and in revised form, January 10, 2012 Published, JBC Papers in Press, January 19, 2012, DOI 10.1074/jbc.M111.324855

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Background: Substrate binding and transport mechanisms of mitochondrial carriers are inadequately understood.
Results: The effect of mutations on substrate specificity and transport activity was assessed in two human ornithine carrier isoforms.
Conclusion: The substrate specificity and transport rate of the two isoforms are defined by a few residues and can be swapped.
Significance: The results show how small substrates can trigger transport in carriers.

Mitochondrial carriers are a large family of proteins that transport specific metabolites across the inner mitochondrial membrane. Sequence and structure analysis has indicated that these transporters have substrate binding sites in a similar location of the central cavity consisting of three major contact points. Here we have characterized mutations of the proposed substrate binding site in the human ornithine carriers ORC1 and ORC2 by carrying out transport assays with a set of different substrates. The different substrate specificities of the two isoforms, which share 87% identical amino acids, were essentially swapped by exchanging a single residue located at position 179 that is arginine in ORC1 and glutamine in ORC2. Altogether the substrate specificity changes demonstrate that Arg-179 and Glu-180 of contact point II bind the C_α carboxylate and amino group of the substrates, respectively. Residue Glu-77 of contact point I most likely interacts with the terminal amino group of the substrate side chain. Furthermore, it is likely that all three contact points are involved in the substrate-induced conformational changes required for substrate translocation because Arg-179 is probably connected with Arg-275 of contact point III through Trp-224 by cation-π interactions. Mutations at position 179 also affected the turnover number of the ornithine carrier severely, implying that substrate binding to residue 179 is a rate-limiting step of the catalytic transport cycle. Given that Arg-179 is located in the vicinity of the matrix gate, it is concluded that it is a key residue in the opening of the carrier to the matrix side.

The inner membrane of mitochondria contains a number of proteins that are responsible for the transport of a variety of metabolites, nucleotides, and cofactors back and forth (1, 2). They belong to the mitochondrial carrier protein family (Pfam PF00153), which in humans comprises about 50 members (3, 4). Sequences of mitochondrial carriers contain highly conserved signature motifs that are used to identify family members in sequence databases (3). The relatively large number of mitochondrial carrier proteins with known substrates (3, 5–7) allowed the identification of a common substrate binding site by using chemical and distance restraints (8), which were derived from homology models based on the structure of the ADP/ATP carrier (9) and by scoring deviations of pseudosymmetry (10). The proposed common substrate binding site is located in the central cavity of the carriers, has three major contact points for the binding and selectivity of the substrates, and enables the classification of the carriers into major groups based on their corresponding substrate classes: nucleotides, carboxylic acids, and amino acids (8).

The transport is proposed to follow a "single binding center gated pore" mechanism (11, 12) that involves the opening and closing of gates on the cytoplasmic and matrix side of the carriers to change the access of the substrate to the common substrate binding site in an alternating way. According to this mechanism, it is the total binding energy of the substrate-carrier interactions in the transition state that induces the conformational changes required for substrate translocation (1, 13, 14). Genetic (15) and structural (9) studies have shown that the matrix gate consists in part of positively and negatively charged residues that form a salt-bridge network in the cytoplasmic state, when the substrate binding site is accessible from the intermembrane space. In analogy, it has been postulated that charged residues on the cytoplasmic side may form a salt-bridge network (cytoplasmic gate) in the matrix state, when the substrate binding site is open to the mitochondrial matrix (10).

* This work was supported by grants from the Ministero dell'Università e della Ricerca (MIUR), the Center of Excellence in Genomics, the Italian Human ProteomeNet Grant RBRN07BMCT_009 (MIUR), and the Medical Research Council (to A. J. R. and E. R. S. K.). The collaboration was funded by the European Community Seventh Framework Programme FP7/2007-2013 under Grant HEALTH-F4-2007-201924, European Drug Initiative on Channels and Transporters (EDICT) Consortium.
[S] This article contains supplemental Figs. 1 and 2.
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Although previous mutagenesis studies have pointed out that residues of the proposed common substrate binding site are critical for function (16–22), no experimental evidence has been reported to show their involvement in determining substrate specificity in mitochondrial carriers. The two characterized isoforms of the ornithine carrier ORC1 [2] and ORC2 form an interesting case because they share 87% identical amino acids but have clear differences in substrate specificity (23). The specificity of ORC1 is restricted to the L-forms of ornithine, lysine, arginine, and citrulline, whereas ORC2 has a much broader specificity, transporting also the D-forms of these amino acids as well as L-histidine and L-homoarginine. The tissue distribution of the isoforms is overlapping; they are found in most tissues with ORC1 expressed more than ORC2 especially in liver, pancreas, lung, and testis (23). Mutations of the ORC1 gene that inactivate the carrier protein and cause the autosomal recessive hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM238970) have provided clues for the functional importance of certain residues (23–27). Some of the missense mutations causing HHH syndrome, such as E180K (24), G220R (25), R275Q (23, 26), G27R, A70L, F188L, G216S, T272I, and L283F (27), affect residues protruding into the substrate translocation pathway of ORC1, suggesting that they might affect substrate binding (8, 13, 28).

In this study, we have shown that the substrate specificity of the human mitochondrial ornithine transporters ORC1 and ORC2 can be manipulated by specific mutations in the proposed common substrate binding site, providing experimental evidence for the involvement of specific residues in substrate selection and binding. The participation of these residues in the ORC1 and ORC2 substrate binding site is further supported by computational docking of ligands to the carriers. The results provide insight into the coupling of substrate binding to the transport mechanism and the determinants of substrate specificity in members of the mitochondrial carrier family.

EXPERIMENTAL PROCEDURES

Sequence and Structure Analysis—Databases were screened with the sequences of ORC1 (GenBank™/European Bioinformatics Institute (EBI), accession number AF112968, gene name *SLC25A15*) and ORC2 (GenBank/EBI, accession number AF332005, gene name *SLC25A2*) with BLASTP to search for homologues. The amino acid sequences were aligned with Multiple Aligner ClustalW in STRAP (29). Comparative models of the ORC1 and ORC2 transporters were built based upon the x-ray-derived structure of the bovine ADP/ATP transporter by using the MODELLER program (30) and checking for steric clashes and poor contacts by using the MolProbity structure evaluation program (31). The substrates transported by each of the two isoforms were docked 100 times into the cavity of ORC1 and ORC2 structural models at pH 7 by using the GOLD program with default parameters (32). Restraints were applied to favor docking solutions in which the ligands interacted with Glu-77 in ORC1 and both Glu-77 and Gln-179 in ORC2. It was not specified which ligand atoms should interact with the protein. The side chains of Glu-77, Arg-179/Gln-179, and Glu-180 were allowed to take up conformations as defined in the penultimate rotamer library (33). The GoldScore scoring potential was used to rank the docking solutions.

Construction of Expression Plasmids—The coding sequences for ORC1 and ORC2 were amplified from human liver cDNA by PCR and were cloned into the pRUN expression vector (23). Site-directed mutations were constructed with overlapping PCR, and the constructs were transformed into *Escherichia coli* TG1 cells (Invitrogen). Transformants were selected on LB (10 g/liter Tryptone, 5 g/liter yeast extract, 5 g/liter NaCl, pH 7.4) plates containing 100 μg/ml ampicillin, and all constructs were confirmed by DNA sequencing.

Bacterial Expression and Purification—ORC1, ORC2, and the mutants were overexpressed as inclusion bodies in the cytosol of *E. coli* CO214(DE3) as described before (34–36). Inclusion bodies were purified on a sucrose density gradient and were washed at 4 °C, first with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.2), then once with a buffer containing 3% Triton X-114 (w/v), 1 mM EDTA, and 10 mM HEPES, pH 7.2, and finally four times with TE buffer. The inclusion body proteins were solubilized in 1.8% sarkosyl (w/v). Unsolubilized material was removed by centrifugation (15,300 × *g* for 10 min), and the supernatant was diluted 1:10 with 5 mM HEPES, pH 7.2, and 0.6% Triton X-114. Homogeneity of the purified wild-type ORC1, wild-type ORC2, and mutant proteins was confirmed by SDS-PAGE.

Reconstitution into Liposomes—The solubilized recombinant proteins were reconstituted into liposomes (37). The reconstitution mixture contained solubilized proteins (1–3 μg), 1% Triton X-114, 1% egg yolk phospholipids as sonicated liposomes, 20 mM substrate, 10 mM HEPES, pH 7.2, 0.6 mg of cardiolipin (Sigma), and water to a final volume of 700 μl. These components were mixed thoroughly, and the mixture was recycled 13 times through a Bio-Beads SM-2 column (Bio-Rad).

Transport Assays—External substrate was removed from proteoliposomes on Sephadex G-75 columns pre-equilibrated with 10 mM HEPES and 50 mM NaCl, pH 7.2. Transport at 25 °C was initiated by adding L-[^{3}H]ornithine (American Radiolabeled Chemicals) to the substrate-loaded proteoliposomes. Transport was terminated by adding 15 mM pyridoxal 5′-phosphate and 18 mM bathophenanthroline according to the "inhibitor-stop" method (37). In controls, the inhibitors were added at the beginning with the radioactive substrate. Finally, the external substrate was removed, and the radioactivity in the liposomes was measured (37). The experimental values were corrected by subtracting control values. The initial transport rates were calculated from the radioactivity taken up by proteoliposomes in the initial linear range of substrate uptake, taking into account the efficiency of reconstitution (*i.e.* the yield of successfully incorporated protein).

Other Methods—Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue dye. The amount of purified ORC1, ORC2, and mutants was estimated by laser densitometry of stained samples using carbonic anhydrase as protein standard (38, 39). The amount of protein incorporated into liposomes was measured as described previously (38, 39) and ranged between 15 and 26% of the protein added in the reconstitution mixture.

[2] The abbreviations used are: ORC, ornithine carrier; HHH, hyperornithinemia-hyperammonemia-homocitrullinuria.

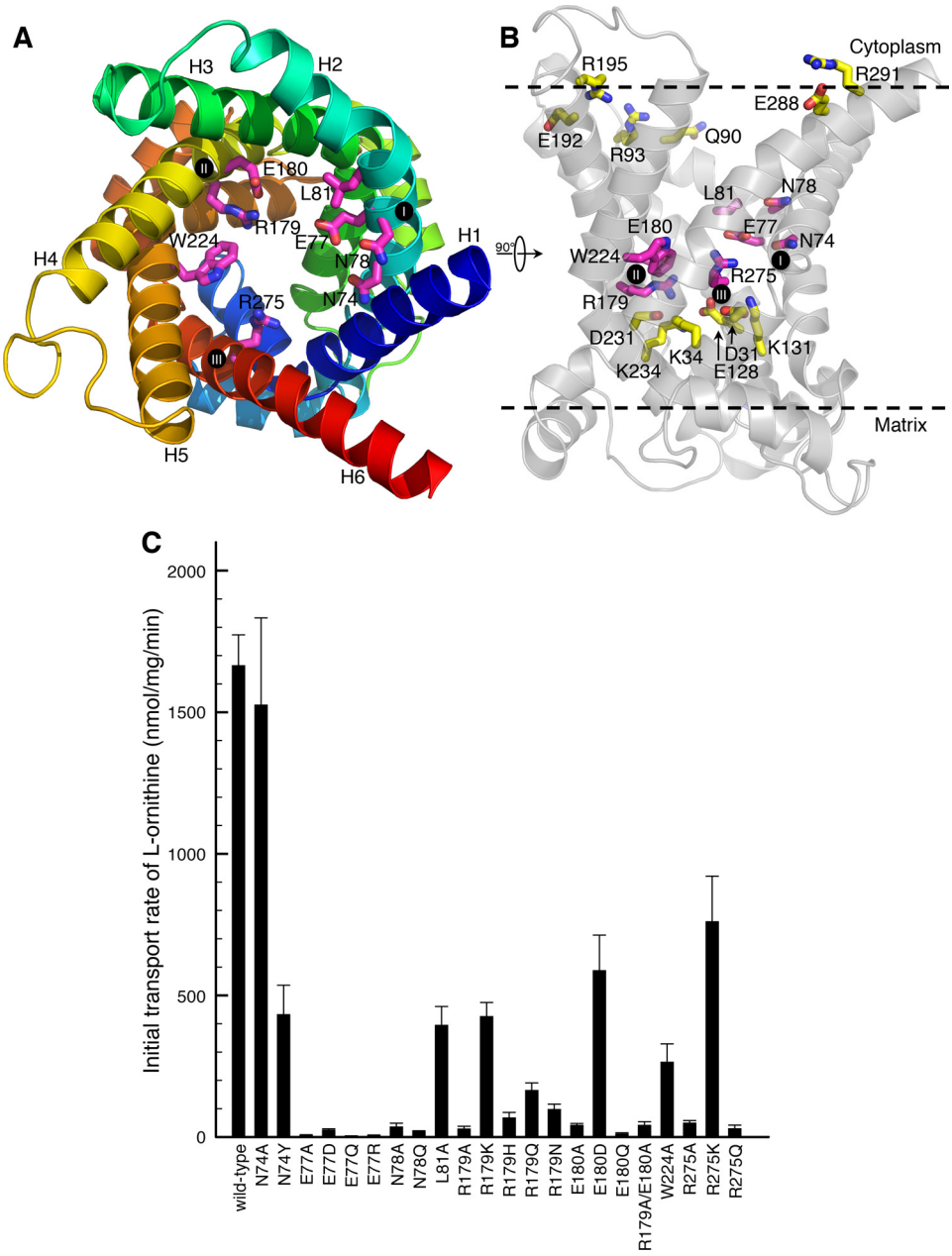

FIGURE 1. **Mutations in proposed ORC1 substrate binding site.** *A* and *B*, the homology model of ORC1, which was built on the structure of the carboxyatractyloside-inhibited ADP/ATP carrier (9), viewed into the cavity from the cytosolic side (*A*) and from the lateral side (*B*). Contact points I, II, and III are indicated. The residues that were mutated are indicated in *magenta*; the positively and negatively charged residues of the cytosolic and matrix gates are indicated in *yellow*. *C*, initial transport rates of [^{3}H]ornithine/ornithine homo-exchange in liposomes reconstituted with wild-type ORC1 and ORC1 mutants. Proteoliposomes were preloaded internally with 20 mM ornithine, and transport was initiated by addition of 0.2 mM [^{3}H]ornithine. The bars represent the mean values of at least three independent experiments, and the *error bars* show the S.E.

RESULTS

Searching for Residues in ORC1 Involved in Substrate Binding—We have investigated the residues of the proposed substrate binding site in human ORC1 and ORC2 by a site-directed mutagenesis approach combined with transport assays. Initially, alanine substitutions were engineered of the ORC1 residues located in or near the proposed common substrate binding site of the mitochondrial carriers (8). These residues protrude into the central cavity of ORC1 at approximately the midpoint of the membrane (Fig. 1, *A* and *B*) and between the matrix and cytosolic salt-bridge networks that have been proposed to act as gates in the substrate translocation mechanism (13, 14, 40). The ORC1 wild-type and mutant carriers were used in ornithine homo-exchange experiments to determine the activity of the proteins by initial transport rate measurements (Fig. 1*C*). For the alanine replacement mutants that were inactive, additional substitutions were made with other amino acids, and their transport activity was quantified (Fig. 1*C*).

N74A exhibited unchanged initial rate of ornithine-ornithine exchange as compared with wild-type, indicating that asparagine at this position is not essential for transport. In contrast, all of the tested mutations of Glu-77 and Asn-78, as well as the nonconservative substitutions R179A, E180A, E180Q, R275A, and R275Q, showed severe detrimental effects on

transport activity. These results demonstrate that the residues Glu-77, Asn-78, Arg-179, Glu-180, and Arg-275 are critical for transport, which can be interpreted as being indispensable for substrate binding and/or conformational changes of the carrier during the catalytic transport cycle. Furthermore, the conservative mutations L81A, R179K, E180D, and R275K caused reduced initial uptake rates for ornithine transport as compared with wild-type, indicating that the particular properties of the side chains of these amino acid positions are required for transport.

The two charged residues Arg-179 and Glu-180 are neighbors both in sequence and in the structural model (Fig. 1*A*) and could potentially form a salt bridge. This observation led us to make a double alanine mutant of these residues to rule out the possibility that the single mutants are detrimental to the activity by leaving the other charge unpaired. However, the double mutant R179A/E180A did not rescue the low activity of the single mutants, giving no experimental support for the interaction suggested above. In addition, the nonconservative ORC1 mutations N74Y and R179Q exhibited a low but significant activity. This finding is especially interesting because tyrosine and glutamine are found at the same positions, respectively, in the human ORC2 isoform. Finally, the nonconservative mutant W224A displayed activity but at a reduced level, which probably means that this residue is involved but not essential for ornithine homo-exchange.

Identification of Residues Determining Substrate Specificity of ORC1—The mutants with low but significant ornithine homo-exchange activity (above 100 nmol/min/mg) were further characterized by hetero-exchange transport of a set of different substrates loaded in the proteoliposomes against externally added radioactive L-ornithine to determine the substrate specificity (Fig. 2). Under the experimental conditions used, the ornithine carrier functions as a strict 1:1 antiporter (41) and, therefore, the L-[^{3}H]ornithine uptake rates reflect the exit rates of the internal substrates as compared with the unaffected L-ornithine homo-exchange rates.

With the N74A, L81A, and R275K mutant proteins, the initial transport rates of L-ornithine, L-arginine, and L-lysine were proportional to those measured with wild-type ORC1. This observation indicates that these mutants have a similar substrate specificity as the wild-type protein. Therefore, the residues Asn-74, Leu-81, and Arg-275 may not bind to the substrate, and if they do, they do not discriminate between the investigated substrates. In contrast, the E180D mutant displayed a decreased specificity of L-ornithine *versus* L-arginine and L-lysine as compared with wild-type ORC1. Thus E180D impedes L-ornithine transport but not L-arginine and L-lysine transport. Furthermore, R179K and W224A also exhibited an altered substrate specificity as compared with that of wild-type ORC1. In fact, although the transport rate values of all the substrates measured with R179K and W224A mutant proteins were lower than those measured with wild-type ORC1, the ratios between the transport rate of L-ornithine and the transport rates of L-arginine and L-lysine were higher with R179K and W224A than those obtained with wild-type ORC1. Therefore, arginine in position 179 and tryptophan in position 224 favor the transport of the longer substrates L-arginine and L-lysine with respect to that of L-ornithine.

Investigation of Residues Determining diverse Substrate Specificity of the Two ORC Isoforms—To date, there are only eight ORC1 and ORC2 sequence pairs in mammalian sequence databases. The alignment of these sequences revealed that there are only two residues that differ between the two isoforms in the predicted cavity and that they are completely conserved within each isoform set of sequences (supplemental Fig. 1). These residues are asparagine or tyrosine in position 74 and arginine or glutamine in position 179 in ORC1 and ORC2, respectively. The observation that ORC1-N74Y and ORC1-R179Q are still active, although at a reduced level (Fig. 1), allowed us to investigate whether the residues in positions 74 and 179 were responsible for the differences in substrate specificity between the two isoforms (Fig. 3).

The mutations ORC1-N74Y and ORC2-Y74N did not significantly change the substrate specificity of wild-type ORC1 and ORC2, respectively. In contrast, the substrate specificities of ORC1-R179Q and ORC2-Q179R mutants were markedly altered as compared with those of wild-type ORC1 and wild-type ORC2, respectively. Thus at sharp variance with wild-type ORC1, the specificity of ORC1-R179Q for L-ornithine was decreased with respect to L-arginine, L-lysine, L-homoarginine, D-ornithine, and D-arginine. This mutant exhibited an almost equal initial transport rate for L-ornithine and L-homoarginine and exhibited about half of this rate for D-ornithine and D-arginine. In strong contrast with wild-type ORC2, the ORC2-Q179R mutant displayed an increased specificity for L-ornithine, L-arginine, L-lysine, and D-lysine as compared with L-histidine, L-homoarginine, D-ornithine, and D-arginine. Moreover, the relative transport rates of the ORC2-Q179R mutant for L-ornithine and the other substrates were similar to those determined with ORC1 wild-type. Notably, the initial rate of ornithine homo-exchange catalyzed by the ORC1-R179Q mutant protein was reduced to 10% as compared with that catalyzed by ORC1 wild type, whereas the one catalyzed by the ORC2-Q179R mutant was enhanced 18-fold as compared with that of ORC2 wild-type. The double mutant ORC1-N74Y/R179Q did not differ much from the ORC1-R179Q mutant in activity levels and specificity. Also, the activity and substrate specificity of ORC2-Y74N/Q179R were similar to those of the single ORC2-Q179R mutant. Thus the substrate specificity of the two isoforms could be swapped by exchanging the residues at position 179.

To characterize the role of glutamine in position 179 of ORC2 further, three additional mutants were made: Q179N, Q179K, and Q179H. These mutants displayed a substrate specificity similar to that of wild-type ORC2 (Fig. 4*A*). The role of the tyrosine in position 74 was also investigated further by replacing tyrosine with alanine, valine, lysine, and aspartate in the ORC2-Q179R background. As shown in Fig. 4*B*, ORC2-Y74A/Q179R, ORC2-Y74V/Q179R, and ORC2-Y74D/Q179R did not change the substrate specificity of ORC2-Q179R, apart from a slightly increased L-arginine transport for ORC2-Y74A/Q179R and ORC2-Y74D/Q179R, as well as a more evident relative increase in L-homoarginine transport by ORC2-Y74D/Q179R. The activity of ORC2-Y74K/Q179R was very low (90 ± 20 nmol of

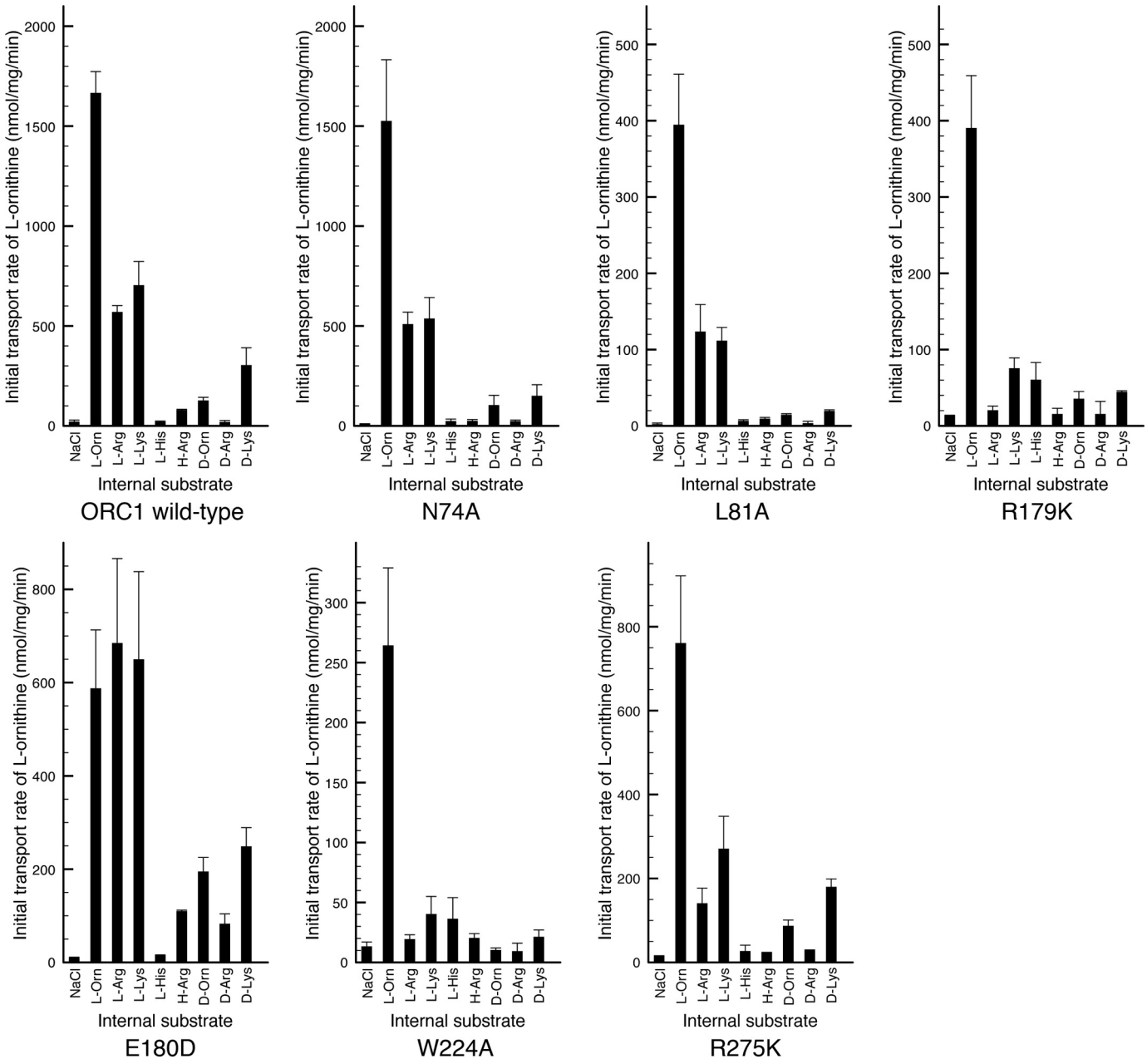

FIGURE 2. **Substrate specificity of wild-type ORC1 and mutants.** Proteoliposomes were preloaded internally with 20 mM of the various substrates indicated: *L-Orn*, L-ornithine; *L-Arg*, L-arginine; *L-Lys*, L-lysine; *L-His*, L-histidine; *H-Arg*, L-homoarginine; *D-Orn*, D-ornithine; *D-Arg*, D-arginine; and *D-Lys*, D-lysine. Transport was initiated by adding 0.2 mM [^{3}H]ornithine. The bars represent the mean values of at least three independent experiments, and the *error bars* show the S.E.

L-ornithine/mg/min) as compared with ORC2-Q179R. Taken together, these results demonstrate the unique effects of arginine in position 179 of ORC2 on the substrate specificity and activity levels of the mitochondrial ornithine transporter.

Kinetic Characterization of ORC Isoform Mutants—The kinetic parameters k_m and V_{max} of most mutants employed in this study were determined for ornithine homo-exchange (Table 1). Many mutations had little effect on the k_m of ORC1 and ORC2. The highest k_m (about 1 mM) was observed with ORC1-N74Y and ORC2-Q179R. It is worth noticing that (i) these mutants contain the combination of the longer side chains Tyr-74 and Arg-179, (ii) the k_m found with the double mutant ORC1-N74Y/R179Q was very similar to that of ORC2 wild-type, and (iii) the k_m observed with ORC2-Y74N/Q179R was similar to ORC1 wild-type. The pronounced increase in initial ornithine transport activity (Fig. 3) of the mutants ORC2-Q179R and ORC2-Y74N/Q179R was reflected in their V_{max} values, which were increased 33- and 12-fold, respectively.

Docking of Substrates in Homology Models of ORC1 and ORC2—Initial computational docking of the substrates to ORC1 and ORC2 produced different binding sites. Thus minimal restraints were applied based on the results of the mutagenesis to find the most likely binding pose. With the restraints indicated under "Experimental Procedures," all the top scoring poses of ORC1 substrates showed the substrate with the C_α carboxylate and amino groups forming salt bridges with Arg-179 and Glu-180, respectively, and the terminal amino group of its side chain forming a salt bridge with Glu-77; 7 of the 10 top

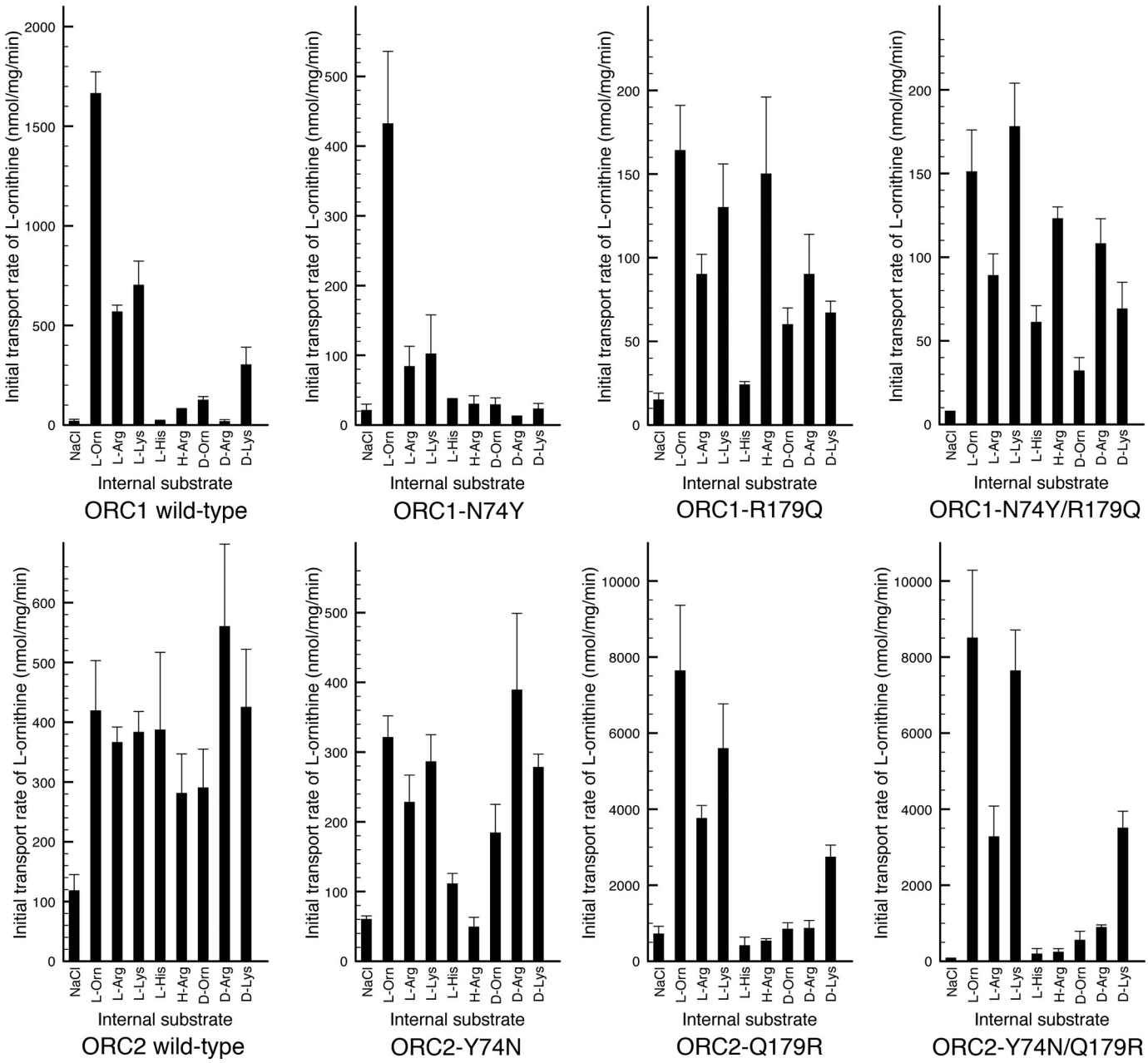

FIGURE 3. **Swap of substrate specificity between ORC1 and ORC2 by exchanging their residues in position 179.** Proteoliposomes were preloaded internally with 20 mM of the various substrates indicated: *L-Orn*, L-ornithine; *L-Arg*, L-arginine; *L-Lys*, L-lysine; *L-His*, L-histidine; *H-Arg*, L-homoarginine; *D-Orn*, D-ornithine; *D-Arg*, D-arginine; and *D-Lys*, D-lysine. Transport was initiated by adding 0.2 mM [^{3}H]ornithine for the ORC1 proteins and 0.4 mM [^{3}H]ornithine for the ORC2 proteins. The bars represent the mean values of at least three independent experiments, and the *error bars* show the S.E.

scoring poses of the ORC2 substrates showed the substrate bound to Glu-77, Gln-179, and Glu-180 in a similar way (Fig. 5 and supplemental Fig. 2).

DISCUSSION

In this study, we have characterized the substrate binding site of the human ornithine carrier isoforms ORC1 and ORC2 by measuring transport activities of site-directed mutants, aiming to reveal the main determinants for substrate specificity. In general, it is difficult to interpret whether a carrier mutation causing an altered initial transport rate, k_m or V_{max}, is affecting the binding of the substrate(s) or the conformational changes occurring during the catalytic transport cycle. Furthermore, some carrier residues are expected to fulfill both roles because it is the total binding energy of the substrate-carrier interactions that triggers the conformational changes needed for substrate translocation (1, 10, 12–14). However, in this work, we have investigated the substrate specificity of the mutant proteins, which helps the interpretation of the mutation-induced effects because an alteration of the substrate specificity suggests that the mutated residue is involved in substrate binding, whereas a significant change in V_{max} alone suggests a role in transport mechanism.

Our transport and docking results are consistent with the model of the ORC1 and ORC2 substrate binding sites depicted

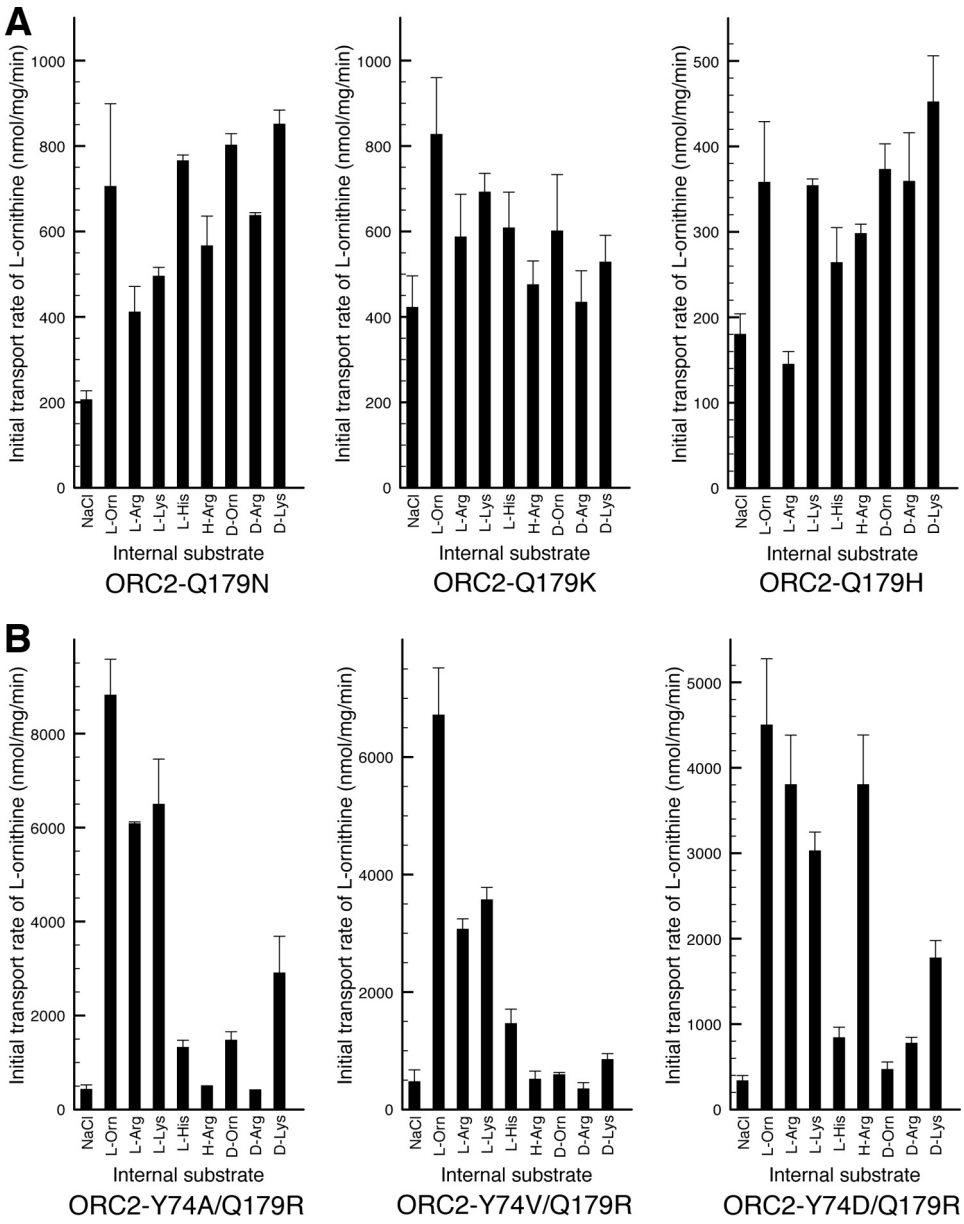

FIGURE 4. **Substrate specificity of ORC2 mutants.** *A* and *B*, proteoliposomes were preloaded internally with 20 mM of the various substrates indicated: *L-Orn*, L-ornithine; *L-Arg*, L-arginine; *L-Lys*, L-lysine; *L-His*, L-histidine; *H-Arg*, L-homoarginine; *D-Orn*, D-ornithine; *D-Arg*, D-arginine; and *D-Lys*, D-lysine. Transport was initiated by adding 0.4 mM L-[^{3}H]ornithine. The bars represent the mean values of at least three independent experiments, and the *error bars* show the S.E.

in Fig. 5 and supplemental Fig. 2, respectively. The molecular details of these models should be taken with caution because the structures of ORC1 and ORC2 are based on the conformation of the carboxyatractyloside-inhibited ADP/ATP carrier, which is open to the cytoplasmic side (9), whereas our measured transport activities not only reflect the substrate binding but also the energy of the interactions in the transition state, in which the substrate is secluded from both the cytoplasmic and the matrix side by partial or complete closure of the cytosolic and matrix gates (1, 6, 10, 13, 14).

Conservative substitutions of residues Arg-179 and Glu-180 lead to altered substrate specificity, whereas nonconservative replacements cause inactivation of the protein (Fig. 2), demonstrating that these residues are involved in substrate binding. Lysine at position 179 in ORC1 makes the protein more specific for L-ornithine, whereas arginine in this position, as in the wild-type, widens the substrate specificity to L-arginine and L-lysine (Fig. 2). It is also clear that the positively charged residue in position 179 is selecting substrates based on their enantiomeric form because when position 179 is occupied by arginine (wild-type ORC1 and ORC2-Q179R, Fig. 3), only the L-forms of ornithine, arginine, and lysine are transported, and when it is occupied by glutamine (wild-type ORC2 and ORC1-R179Q, Fig. 3), also the D-forms of these amino acids are transported. The most straightforward explanation of these findings is that the carboxyl group of the chiral center C_α of the substrate binds to the residue in position 179 and that its amino group interacts with Glu-180, which is protruding into the cavity next to Arg-179 in wild-type ORC1.

The E180D mutation showed a decreased specificity for the shorter substrate L-ornithine with respect to L-lysine and L-ar-

TABLE 1

Kinetic constants of ORC1, ORC2, and mutant proteins

The values were calculated from double reciprocal plots of the rate of [^{3}H]ornithine/ornithine homo-exchange *versus* substrate concentrations in liposomes reconstituted with the indicated recombinant proteins. Proteoliposomes were preloaded internally with 20 mM ornithine, and transport was initiated by adding different concentrations of [^{3}H]ornithine ranging from 0.02 and 2.0 mM for ORC1 and ORC1 mutants to from 0.04 and 2.0 mM for ORC2 and ORC2 mutants. The data represent the mean values of at least three independent experiments ± the standard errors.

	K_m	V_{max}
	mM	*nmol/min/mg*
ORC1[a]		
Wild-type[a]	0.22 ± 0.02	3000 ± 400
N74A	0.27 ± 0.06	900 ± 200
N74Y	1.0 ± 0.1	750 ± 200
L81A	0.14 ± 0.01	900 ± 100
R179K	0.37 ± 0.07	2800 ± 100
R179Q	0.13 ± 0.05	220 ± 30
E180D	0.07 ± 0.02	220 ± 70
N74Y/R179Q	0.33 ± 0.05	940 ± 80
ORC2[a]		
Wild-type[a]	0.40 ± 0.06	1200 ± 200
Y74N	0.63 ± 0.01	420 ± 10
Q179R	1.1 ± 0.3	40,000 ± 5000
Q179N	0.36 ± 0.07	2300 ± 100
Y74N/Q179R	0.2 ± 0.1	14,000 ± 1000
Y74A/Q179R	0.54 ± 0.03	29,000 ± 3000
Y74V/Q179R	0.79 ± 0.05	30,000 ± 5000
Y74D/Q179R	0.35 ± 0.06	10,000 ± 2000

[a] Values determined in Ref. 23.

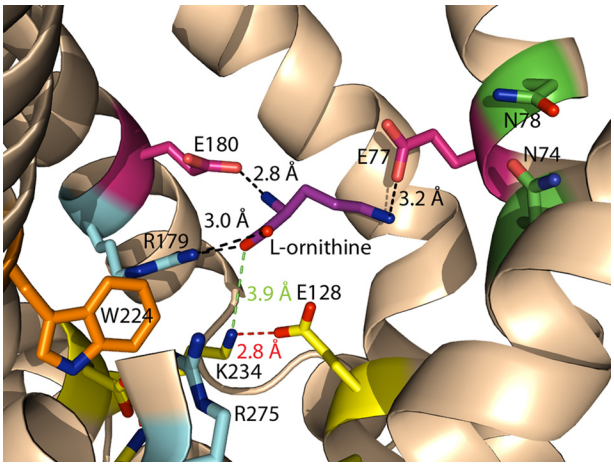

FIGURE 5. **ORC1 substrate binding site.** L-Ornithine (*purple*) is bound to residues Arg-179, Glu-180, and Glu-77 of the ORC1 model. The carrier is shown side-on in the plane of the membrane, and transmembrane helix H6 has been removed from the foreground to expose the cavity. Salt bridges between the substrate and carrier are shown as *black dashed lines*. Interactions of the salt-bridge network at the base of the cavity are shown by *red dashed lines*. The close proximity of L-ornithine to residue Lys-234 of the salt-bridge network (the matrix gate) is shown as a *green dashed line*. Residues Asn-74, Asn-78, and Trp-224 do not interact with the substrate, but Trp-224 constrains the orientation of Arg-179. Salt-bridge residues (*yellow*), plus basic (*cyan*), acidic (*magenta*), other polar (*green*), aromatic (*orange*), and aliphatic (*pink*) residues are shown.

ginine as compared with wild-type ORC1 (Fig. 2). This observation suggests that the mutation repositions the substrate toward position 180, as a consequence of the glutamate to aspartate substitution, and thereby the interaction between the substrate carboxylate group and Arg-179 is skewed and maybe the terminal amino group of the shorter substrate ornithine becomes more distant from its binding partner on the other side of the cavity. Supporting evidence for the importance of position 180 comes from the E180K mutation in ORC1 that causes HHH syndrome (24).

Although Arg-179 is highly conserved among all carriers transporting amino acids and carboxylic acids (8, 13), in the ORC subfamily, the residue at position 179 displays a co-variation with that in position 74; in ORC1, the pair is Asn-74 and Arg-179, whereas in ORC2, it is Tyr-74 and Gln-179 (supplemental Fig. 1). Asn-74 is not crucial for substrate binding because the ORC1-N74A mutant displayed similar specificity and activity as the wild-type (Figs. 1 and 2), and Tyr-74 in ORC2 is also not essential because it can be substituted with various residues in the ORC2-Q179R background without a major effect on the transport characteristics (Fig. 4). Interestingly, when the pair is a hybrid formed by tyrosine in position 74 and arginine in 179, the k_m of ornithine is increased to about 1 mM in both cases (ORC1-N74Y and ORC2-Q179R, Table 1). The most likely explanation is that this hybrid combination negatively affects the size and shape of the substrate binding site pocket in the transition state. This hypothesis is further supported by the fact that the double mutations in positions 74 and 179 (ORC1-N74Y/R179Q and ORC2-Y74N/Q179R) restore the k_m of the wild-type into which they have been transformed (Table 1).

Residues Glu-77 and Asn-78, which are located on transmembrane helix H2, as is Asn-74, are crucial for the function of the ornithine carrier as none of the tested substitutions in these positions were active (Fig. 1). Glutamate at position 77 and asparagine at position 78 are specific for ORC transporters, although they are not completely conserved in all ORC2 sequences (supplemental Fig. 1). It is likely that Glu-77, located on the opposite side of the carrier cavity with respect to Arg-179 and Glu-180, interacts with the positively charged amino group of the side chain of the substrates, positioning them across the carrier cavity. Asn-78 might play a role in guiding the ligands to Glu-77, which is deeper in the cavity.

The W224A substitution leads to a carrier protein that is more specific for L-ornithine with respect to L-arginine and L-lysine (Fig. 2). A tryptophan is also found in other mitochondrial carriers in the equivalent position of Trp-224: the carnitine/acyl-carnitine transporters (42–46); the yeast Ymc1p and Ymc2p, which are implicated in oleic acid and glutamate metabolism (47); and the *Arabidopsis thaliana* basic amino acid carriers BAC1 and BAC2 (48, 49) (see alignments in Refs. 6, 8, and 13). The docking studies suggest that Trp-224 does not interact directly with the ligands. The altered specificity caused by replacement of Trp-224 with alanine can be explained by the potential of Trp-224 to form cation-π interactions with Arg-179 and Arg-275, thereby constraining Arg-179 for substrate binding and mediating substrate-induced conformational changes to Arg-275, which is in contact point III of the proposed substrate binding site. Thus the asymmetric binding of the small substrate in the central cavity would lead to a symmetric transport mechanism by involving all three contact points simultaneously.

All of the nonconservative substitutions of Arg-275 abolish transport activity, which is consistent with the previous observation that R275Q causes HHH syndrome (26) and renders the ORC1 protein inactive (23). It should be noted that arginine at position 275 is highly conserved in almost all subfamilies of mitochondrial carriers (8, 13). It corresponds to Arg-288 of the bovine oxoglutarate carrier (OGC), to Arg-294 of the yeast

ADP/ATP carrier 2 (AAC2), and to Arg-278 of the murine uncoupling protein 1 (UCP1), which also do not tolerate replacement with other amino acids (Ref. 28 and references therein). Arg-275 also corresponds to Arg-588 of the human aspartate/glutamate carrier 2 (AGC2); its substitution with glutamine causes type II citrullinemia (50). Therefore, arginine at this position might have a key role in the transport mechanism of mitochondrial carriers.

In agreement with the hypothesis that mitochondrial carriers have a similarly located substrate binding site (8) and a conserved transport mechanism, it was found that the bovine 2-oxoglutarate carrier, another member of the mitochondrial carrier family (51–53), is inactivated by cysteine replacement of the residues corresponding exactly to those characterized here (ORC1, Glu-77, Asn-78, Arg-179, Glu-180, and Arg-275; 2-oxoglutarate carrier, Tyr-94, Thr-95, Arg-190, Ala-191, and Arg-288) (16, 54). In the carnitine/acyl-carnitine carrier, the residues Arg-178, Asp-179, and Arg-275, which correspond to Arg-179, Glu-180, and Arg-275 in ORC1, were also shown to be important for transport (17). However, in this case, the double alanine mutant R178A/D179A rescued the lack of activity of the single mutants, albeit to a small extent (17), unlike the ORC1-R179A/E180A mutant (this study), which might reflect requirement variations in different mitochondrial carriers to bind their specific substrates. In addition, replacement of Arg-181 in the citrate carrier, corresponding to Arg-179 in ORC1, was found to be deleterious for citrate transport activity (19).

In this work, we have demonstrated that the residue at position 179 in the two human ORC isoforms is largely responsible for the difference in their substrate specificity (Figs. 2 and 3). This position is also important for the transport mechanism. In fact, ORC1 harboring Arg-179 displays a V_{max} value 2.5-fold higher than ORC2 harboring Gln-179; mutant ORC1-R179Q displays a V_{max} value 14-fold lower than wild-type ORC1; and mutant ORC2-Q179R displays a V_{max} value 33-fold higher than wild-type ORC2 (Table 1). This means that the turnover numbers are: 5500 s^{-1} for wild-type ORC1, 2200 s^{-1} for wild-type ORC2, 400 s^{-1} for ORC1-R179Q, and 73,000 s^{-1} for ORC2-Q179R. Clearly, the total substrate-carrier binding energy (which triggers the conformational change leading to substrate translocation) largely depends on the mitochondrial ornithine carrier residues at position 179. We infer that the residue at position 179 plays an important role in determining the rate of the mitochondrial carrier conformational change and therefore the carrier turnover number. The vicinity of Arg-179 to the charged residues of the matrix gate (Fig. 5) suggests that Arg-179 is involved directly or through the bound ligand in the substrate-induced perturbation of the salt-bridge network leading to the opening of the matrix gate and allowing substrate progression toward the matrix.

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