Functional Domains in the Carnitine Transporter OCTN2, Defective in Primary Carnitine Deficiency*

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Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation characterized by hypoketotic hypoglycemia and skeletal and cardiac myopathy. It is caused by mutations in the Na+-dependent organic cation transporter, OCTN2. To define the domains involved in carnitine recognition, we evaluated chimeric transporters created by swapping homologous domains between OCTN1, which does not transport carnitine, and OCTN2. Substitution of the C terminus of OCTN2 (amino acid residues 342–557) with the corresponding residues of OCTN1 completely abolished carnitine transport. The progressive substitution of the N terminus of OCTN2 with OCTN1 resulted in a decrease in carnitine transport associated with a progressive increase in the $K_m$ toward carnitine from 3.9 ± 0.5 to 141 ± 15 μM. A large drop in carnitine transport (and in $K_m$ toward carnitine) was observed with the substitution of residues 341–454 of OCTN2. An additional chimeric transporter (CHIM-9) in which only residues 341–454 of OCTN2 were substituted by OCTN1 had markedly reduced carnitine transport, with an elevated $K_m$ toward carnitine (63 ± 5 μM). Site-directed mutagenesis and introduction of residues nonconserved between OCTN1 and OCTN2 in the OCTN2 cDNA indicated that the R341A, L409W, L424Y, and T429I substitutions significantly decreased carnitine transport. Single substitutions did not increase the $K_m$ toward carnitine. By contrast, the combination of three of these substitutions (R341W + L409W + T429I) greatly decreased carnitine transport and increased the $K_m$ toward carnitine (20.2 ± 4.5 μM). The Arg-341, Leu-409, and Thr-429 residues are all located in predicted transmembrane domains. Involvement of these residues in carnitine transport was further supported by the partial restoration of carnitine transport by the introduction of these OCTN2 residues in the OCTN1 portion of CHIM-9. These studies indicate that multiple domains of the OCTN2 transporter are required for carnitine transport and identify transmembrane residues important for carnitine recognition.

Primary carnitine deficiency (On-line Mendelian Inheritance in Man 212140) is a recessively inherited disorder of fatty acid oxidation due to defective carnitine transport (1). Carnitine is essential for the transfer of long-chain fatty acids from the cytosol to mitochondria for subsequent β oxidation and the lack of carnitine impairs the ability to use fat as fuel during periods of fasting or stress. This can result in hypoketotic hypoglycemia, Reye’s syndrome, and sudden infant death in younger children or in skeletal or cardiac myopathy with insidious onset later in life (1).

The gene for primary carnitine deficiency, SLC22A5, encodes the carnitine transporter OCTN2 (2, 3), and several mutations have been identified in affected patients (reviewed in Refs. 4 and 5). OCTN2 is a novel organic cation transporter and operates a sodium-dependent transport of carnitine and a sodium-independent organic cation transport (3, 6, 7). This transporter was originally cloned for its homology to hOCTN1 (8), the sequence of which is 88% homologous and 77% identical to that of hOCTN2 (2, 3, 8). Unlike OCTN2, hOCTN1 does not transport carnitine. Sodium-dependent carnitine transport mediated by OCTN2 is electrogenic; 1 sodium ion enters the cell with 1 molecule of carnitine (9, 10).

Genes homologous to hOCTN1 and hOCTN2 have been identified in the rat and the mouse. The corresponding transporters have recognition and functional properties similar to those reported for hOCTN1 and hOCTN2 (7, 11–13). A novel carnitine transporter, OCTN3, has been identified in the mouse (13). This transporter has high homology to OCTN1 and OCTN2, mediates a low-affinity sodium-independent carnitine transport, and is expressed only in the mouse testis (13). The corresponding human and rat genes have not yet been identified. A third carnitine transporter, CT2, was isolated from human testis (14). This latter transporter has high affinity toward carnitine ($K_m$ 20 μM) but is only partially sodium-dependent (14). The structure of this transporter is only partially conserved with OCTN1 and OCTN2 (14). Its exclusive presence in the testis limits its physiological role in other tissues (14). Finally, the amino acid transporter ATB3, which is unrelated to the organic cation family of membrane transporters, can transport carnitine with low affinity ($K_m$ 0.8 mM) (15). This transporter is expressed primarily in the lungs, mammary gland, and the intestine. This latter characteristic might explain residual intestinal carnitine transport in patients with primary carnitine deficiency (15).

Although the pharmacological and recognition properties of OCTN2 have been investigated in many systems, little is known about the domains and residues of this transporter involved in carnitine and sodium recognition. The study of natural mutations identified in patients with primary carnitine deficiency has identified domains essential for carnitine (as opposed to organic cation) transport located in transmembrane domain 11 (P478L and S467C mutations) (16, 17). This domain was proposed to contain the anionic binding site for carnitine

* This work was supported by Grant DK 53824 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: OCTN, organic cation transporter; hOCTN, human OCTN; CHO, Chinese hamster ovary.
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EXPERIMENTAL PROCEDURES

Cell Culture and Carnitine Transport—Chinese hamster ovary (CHO) cells were grown in Ham’s F12 medium supplemented with 6% fetal bovine serum. Carnitine transport was measured at 37 °C with the cells plated as described previously (10, 18). Cells were grown to confluence in 24-well plates (Costar) and depleted of intracellular water content, and transport velocity was normalized for intracellular water content. Nonsaturable carnitine transport, measured in the presence of 2 mM cold carnitine from total transport (10, 18). The two approaches gave similar results (10). Values are reported as means ± S.E. of 3–6 independent determinations. Preliminary experiments indicated that carnitine transport is linear up to 30 min in cells expressing the normal OCTN2 transporter (10).

For sodium-independent transport, methylglucamine chloride was substituted for sodium chloride in the extracellular medium (10). To study kinetic constants for carnitine transport were determined by nonlinear regression analysis according to a Michaelis-Menten equation (18). Nonsaturable carnitine transport, measured in the presence of 2 mM carnitine, was subtracted from total transport to obtain saturable carnitine transport (10). Nonlinear parameters are expressed as means ± S.D. Comparisons for significance were performed using 95% (p < 0.05) or 99% (p < 0.01) confidence intervals.

In previous studies (10), we used multiple carnitine concentrations to define the Kₘ toward carnitine (K_mC). However, we noted that the value obtained from the intercept of multiple regression was indistinguishable from those published previously for these cells (10). This is because, in a random bireactant system such as the carnitine/sodium cotransporter, the apparent K_mC toward the co-substrate approaches the true K_mC (K_mC) as the concentration of the substrate (carnitine) decreases to near zero (19). Because our previous studies indicated experimentally that a concentration of 0.5 μM already gave values of apparent K_mC indistinguishable from those calculated from the intersection of multiple curves, in this study we used an even lower concentration of carnitine (0.1 μM) to obtain an apparent K_mC that closely approaches true K_mC. As shown in the results below, the values obtained with this system are similar or identical to those published previously for these cells (10).
OCTN2 was substituted by OCTN1, the $K_m$ toward carnitine increased to 5.2 ± 0.8 μM (no significant change versus OCTN2) in CHIM-1, 9.8 ± 0.8 μM in CHIM-2 ($p < 0.01$ as compared with wild-type OCTN2 using 99% confidence intervals), 12.5 ± 0.8 μM in CHIM-3 ($p < 0.01$), and 141 ± 19 μM in CHIM-4 ($p < 0.01$). By contrast, there were only minimal changes in $V_{\text{max}}$ among most transfectants. The $V_{\text{max}}$ was 111 ± 6 nmol/ml cell water/h with OCTN2, 105 ± 4 nmol/ml cell water/h with CHIM-1 (not significantly different from OCTN2), 108 ± 2 nmol/ml cell water/h with CHIM-2 (not significantly different from OCTN2), 133 ± 4 nmol/ml cell water/h with CHIM-3 ($p < 0.05$ versus OCTN2), and 215 ± 20 nmol/ml cell water/h with CHIM-4 ($p < 0.01$ versus OCTN2).

Analysis of cells expressing CHIM-5 indicated a near normal $K_m$ toward carnitine (10.6 ± 3.9 μM, not significantly different from OCTN2) with a markedly reduced $V_{\text{max}}$ (18.1 ± 2 nmol/ml cell water/h, $p < 0.01$ versus OCTN2). The near normal $K_m$ of CHIM-5 indicates that the majority of residues needed for...
carnitine recognition are contained in the first 453 amino acids of OCTN2. This further supported the results obtained with CHIM-1, CHIM-2, CHIM-3, and CHIM-4 indicating that the substitution of the first 453 amino acids of OCTN2 with the corresponding portion of OCTN1 caused a progressive reduction in the affinity for carnitine. The sudden increase in $K_m$ between CHIM-3 and CHIM-4 indicated that a major domain required for carnitine recognition was contained within residues 341 and 453 of OCTN2.

Sodium Stimulation of Carnitine Transport in CHO Cells Expressing Normal and Chimeric OCTN Transporters—The binding of a co-substrate can affect the affinity of a transporter toward the substrate (19). We have previously seen that a marked decrease in extracellular sodium concentration increases the $K_m$ of OCTN2 toward carnitine (10). Kinetic analysis of carnitine transport at different extracellular sodium concentrations indicated that sodium has at least two functions in carnitine transport (10). At low concentrations, sodium lowers the $K_m$ of OCTN2 toward carnitine (10), suggesting binding to a site close to the carnitine binding site. Higher sodium concentrations provide the electrochemical gradient to transfer the carnitine-sodium complex inside the cell (10).

To exclude that the changes in the $K_m$ toward carnitine measured in CHIM-1, CHIM-2, CHIM-3, and CHIM-4 were caused by abnormal interaction with the co-transported sodium, the kinetics of sodium-stimulated carnitine transport were obtained (Fig. 4). Half-maximal stimulation of carnitine transport was obtained at a sodium concentration of 13 ± 2.7 mM in CHO cells expressing the normal OCTN2 cDNA, a value similar to that reported previously in CHO cells (11.6 mM, Ref. 10) and in human fibroblasts that express the same transporter (11.4 ± 2.1 mM, Ref. 18). $K_{Na}$ (the concentration of sodium at which half-maximal stimulation of carnitine transport was observed) increased to 51.7 ± 6.6 mM with CHIM-1 ($p < 0.01$ versus OCTN2 using 99% confidence intervals) and remained elevated in CHIM-2 (41.9 ± 3.8 mM, $p < 0.01$ versus OCTN2) and CHIM-3 (38.1 ± 7.9 mM, $p < 0.05$ versus OCTN2). $K_{Na}$ further increased to 90 ± 26 mM in CHIM-4 ($p < 0.05$ versus OCTN2). These results indicate that substitution of the N-terminus of OCTN2 with OCTN1 in CHIM-1 (amino acids

![Fig. 4. Sodium dependence of carnitine transport by CHO cells expressing normal (A) and chimeric OCTN transporters (B–G).](image)
1–193) leads to an increase in $K_{Na}$ toward carnitine seen in CHIM-1 could be secondary to changes in the affinity toward the co-transported sodium rather than reflect primary changes of the carnitine binding site. Additional substitution of the N terminus of OCTN2 with OCTN1 in CHIM-2 and CHIM-3 did not appear to further impair the interaction with sodium, as $K_{Na}$ did not further increase. However, the progressive substitution of OCTN2 with OCTN1 in CHIM-2 and CHIM-3 did result in a significant progressive increase in the $K_{Na}$ toward carnitine (Fig. 3), indicating the presence of domains interacting directly with the substrate between amino acids 194 and 340. Further substitution of OCTN2 with OCTN1 in CHIM-4 results in markedly decreased carnitine transport for both an increase in $K_{Na}$ (90 ± 26 mM) and a dramatic increase in the $K_{Na}$ toward carnitine (141 ± 19 μM). Although multiple domains of the transporter contribute to carnitine recognition, the domain comprised between amino acids 341 and 453 of OCTN2 is the one with the most severe effect on carnitine (and possibly sodium) recognition.

CHIM-5 had a near normal $K_{Na}$ toward carnitine (Fig. 3). However, the $V_{max}$ for carnitine transport was further reduced (Fig. 3). This behavior was similar to that previously described for the E452K mutant carnitine transporter, which had a normal $K_{Na}$ toward carnitine but markedly increased $K_{Na}$ (10). The E452K mutation did not affect the $K_{Na}$ of the transporter toward carnitine and remained normally sensitive to the effects of low concentrations of sodium on the $K_{Na}$ toward carnitine, indicating that it is not located close to a carnitine binding site (10). Therefore, the increased $K_{Na}$ measured with the E452K mutant OCTN2 was likely because of a less efficient coupling of carnitine transfer to the sodium electrochemical gradient (10). In CHIM-5, the domain of OCTN2 comprising Glu-452 is substituted by OCTN1. CHIM-5 had indeed a significantly increased $K_{Na}$ (100 ± 38 mM, $p < 0.05$ versus OCTN2) despite a near normal $K_{Na}$ toward carnitine (Fig. 4). These results further support the hypothesis that the C terminus of the OCTN2 carnitine transporter contains a domain essential for coupling the sodium electrochemical gradient to the transmembrane transfer of the sodium-carnitine complex (10).

Role of Residues 341-451 of OCTN2 in Carnitine Transport—To define the function of the domain comprising amino acids 341–453 of OCTN2 in carnitine transport, one new chimeric transporter, CHIM-9, was produced. In CHIM-9, only amino acids 341–453 of OCTN2 were substituted by the corresponding residues of OCTN1 (Fig. 1). Despite the limited substitution, carnitine transport was reduced to levels similar to those of CHIM-4 (Fig. 2), where the first 453 residues were substituted by OCTN1. This result confirmed the presence of a domain essential for carnitine transport in residues 341–453 of OCTN2.

Kinetic analysis of CHIM-9 indicated that the chimeric transporter had a large increase in the $K_{Na}$ toward carnitine (63 ± 5 μM, $p < 0.01$ versus OCTN2 using 99% confidence intervals; Fig. 3) accompanied to a change in $K_{Na}$ (63.1 ± 5.5 mM, $p < 0.01$ versus OCTN2; Fig. 4). These results confirmed that the domain of OCTN2 comprised between residues 341 and 453 contained residues essential for carnitine recognition and perhaps coupling to the sodium electrochemical gradient.

Effect of Single Amino Acid Substitutions on Carnitine Transport by OCTN2—The domain comprising amino acids 341–453 of OCTN2 contains 25 amino acid residues that are neither identical ($) nor conserved (+) between OCTN1 and OCTN2 (Fig. 5). Rat and mouse OCTN2 transport carnitine in a manner similar to their human counterpart. When the rat, mouse, and human cDNAs for OCTN1 and OCTN2 were aligned, only 14 residues were consistently different between OCTN1 and OCTN2 (Fig. 5, indicated by # symbol). Therefore, we substituted these OCTN1 residues in the OCTN2 cDNA and evaluated their effect on carnitine transport (Fig. 6). Of the substitutions tested, R341A, L409W, L424Y, and T429I were the ones that resulted in the greater impairment of carnitine transport (about 40% of OCTN2 transport activity). When these mutations were combined into a single plasmid, carnitine transport was further reduced to about 10% of wild-type OCTN2 (Fig. 6, last three bars on far right). The greatest reduction was seen when R341A, L409W, and T429I were combined together. The quantitative decrease in carnitine transport further confirmed the importance of these residues in carnitine recognition and coupling to the sodium electrochemical gradient.
transport obtained with R341L, L409W, and T429I was similar to that observed with CHIM-9 in which the whole region containing amino acids 341–453 of OCTN1 was substituted in OCTN2 (Fig. 1). Therefore, these substitutions were likely the major changes between OCTN1 and OCTN2 within residues 341–453 responsible for the abnormal carnitine transport in CHIM-9.

Kinetic constants for carnitine transport by R341L, L409W, L424Y, and T429I mutant OCTN2 transporters are shown in Fig. 7. All of these mutations resulted in a decrease in the $V_{\text{max}}$ for carnitine transport without causing any significant change in the $K_m$ toward carnitine. These results indicated that although changes in single amino acids are important in carnitine transport, they are not sufficient to affect the affinity for carnitine.

We then evaluated the kinetic constants for carnitine transport by cells expressing a combination of mutations. As shown in Fig. 7, the combinations R341A/L409W and R341A/L409W/L424Y further decreased the $V_{\text{max}}$ for carnitine transport but had little effect on the $K_m$ that did not increase significantly above that of OCTN2. In contrast, the combination R341A/L409W/T429I caused a further decline in the $V_{\text{max}}$ and significantly increased the $K_m$ toward carnitine to 20.2 ± 4.5 μM ($p < 0.01$ versus paired OCTN2 using 99% confidence intervals). Therefore, although the substitution of single residues was unable to modify the $K_m$ toward carnitine, the combination of multiple mutations caused a significant change.

CHIM-9 caused an increase in $K_{Na}$ (Fig. 4). We tested OCTN2 constructs with single mutations for their effect on sodium stimulation of carnitine transport (Fig. 8). None of the mutants tested, including R341A, L409W, L424Y, or T429I alone or in combination, caused any significant change in $K_{Na}$ as compared with OCTN2. Specifically, in the triple mutant R341A/L409W/T429I, $K_{Na}$ was 15.6 ± 0.6 mM, a value compa-
rable with that obtained in the paired OCTN2 (17.8 ± 2.8 mM). Therefore, the triple mutant R341A/L409W/T429I decreased carnitine transport without affecting $K_{Na}$, but decreased the $V_{max}$ and increased the $K_m$ toward carnitine, suggesting that the affected residues might be involved selectively in carnitine binding and/or transfer across the plasma membrane.

Restoration of Carnitine Transport by the Introduction of A339R, W407L, and I427T Substitutions in OCTN1 in the Chimeric Transporter CHIM-9—Because the substitution of only 3 residues in OCTN2 with the corresponding residues of OCTN1 had such a profound effect on carnitine transport, we evaluated whether the introduction of OCTN2 residues in the portion of OCTN1 contained in CHIM-9 was capable of restoring carnitine transport. As shown in Fig. 9, the progressive introduction of the A339R, W407L, and I427T (CHIM-9 is 2 residues shorter than OCTN2 in this area, and the numbering is therefore reduced correspondingly) substitutions caused a progressive increase in carnitine transport that reached more than 60% of the paired OCTN2 control.

Kinetic analysis of carnitine transport by cells expressing CHIM-9 with different substitutions indicated that reintroduction of the A339R, W407L, and I427T substitutions in the transmembrane domain resulted in a $K_m$ toward carnitine that was improved as compared with CHIM-9 but not yet normalized (Fig. 10). CHIM-9 had a $K_m$ toward carnitine of 63.2 ± 1.5 μM and the introduction of A339R, W407L, and I427T decreased it to 19.8 ± 1.9 μM ($p < 0.01$ as compared with paired OCTN2 and CHIM-9). The improved $K_m$ was associated with a progressive increase in the $V_{max}$ for carnitine transport. Because CHIM-9 also has a mild but significant effect on $K_{Na}$ (Fig. 4), we evaluated sodium stimulation of carnitine transport in cells expressing CHIM-9 and different amino acid substitutions (Fig. 11). As previously seen, $K_{Na}$ was increased in CHIM-9 (76 ± 7.8 mM, $p < 0.01$ versus paired OCTN2). Substitutions of single or multiple amino acids in CHIM-9 did not significantly improve $K_{Na}$ as compared with CHIM-9. This result confirms that these substitutions selectively modified carnitine recognition, as suggested previously by single amino acid substitutions in OCTN2 (Figs. 6–8), without affecting $K_{Na}$.

**Discussion**

The carnitine transporter OCTN2 is a member of the organic cation transporter family. Unique among these transporters, OCTN2 is sodium-dependent and operates a sodium/carnitine cotransport with 1:1 stoichiometry (9, 10). Very little is known about the portion of OCTN2 (or other similar transporters) required to recognize the substrate or sodium. In this study, we utilized chimeric transporters (Fig. 1) with portions deriving from OCTN2 and OCTN1; the latter is a transporter very similar to OCTN2 that does not transport carnitine. Our results indicate that multiple domains of OCTN2 are required for function.
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Fig. 11. Sodium stimulation of carnitine transport in CHO cells expressing normal (A) and mutant OCTN2 transporters (B–E). Carnitine (0.1 μM) transport was measured for 20 min at 37°C in the presence of increasing concentrations of sodium (0–150 mM). Cells were washed twice with a sodium-free solution prior to transport. Points are means ± S.E. of triplicates. Black lines are the best fitting to a rectangular hyperbola. \( K_m \) is the concentration of sodium at which half-maximal stimulation of carnitine transport is measured.

Substitution of more than 100 amino acids in the C terminus of OCTN2 by OCTN1 abolished carnitine transport activity (Fig. 2). If only about 100 amino acids were substituted (CHIM-9), there was still a marked decrease of carnitine transport (Figs. 2 and 3). The most dramatic decline in function was observed with substitution of residues 341–453, which almost abolished carnitine transport through the transfected transporter. For this reason, we focused our attention on this area of the OCTN2 transporter to define the specific residues responsible for the changes in \( K_m \) and, possibly, that interact with carnitine. A number of amino acids differ between OCTN1 and OCTN2 between residues 341 and 453. However, only 14 are always different between OCTN1 and OCTN2 among mammalian species (Fig. 5). Substitution of these residues indicated that 4 of them caused a substantial decline in carnitine transport (Fig. 6). Further, the combination of different mutations, R341A/L409W/T429I, had a synergistic effect in decreasing carnitine transport to the level seen with substitution of residues 341–453 in CHIM-9 (Fig. 6). The decrease in carnitine transport caused by the combined R341A/L409W/T429I substitutions was associated with an increased \( K_m \) toward carnitine (Fig. 7) with no changes in \( V_{\text{max}} \) (Fig. 8). These results suggest that these residues might interact with carnitine either during recognition or during transmembrane transfer.

To further confirm the importance of these residues in carnitine transport, the OCTN2 amino acids were inserted into the OCTN1 portion of CHIM-9 in these positions (Fig. 9). This procedure almost completely restored carnitine transport and produced a transporter with markedly improved \( K_m \) toward carnitine but requiring an increased amount of sodium to half-maximally stimulate carnitine transport (Figs. 9–11).

OCTN2 has 12 predicted transmembrane domains, with both the N terminus and the C terminus facing the cytoplasm (2, 3). Although there is no experimental evidence for this model, this topology is supported by the homology of this transporter with other organic cation transporters and with evidence from domain-specific antibodies (21). Independently from the model utilized, R341A/L409W and T429I are located in hydrophobic regions that correspond to membrane-spanning domains 7, 9, and 10, respectively. Therefore, our study identifies transmembrane regions as major determinant for carnitine recognition and uptake. This is not unique for this transporter. In organic anion transporters (OAT), which have a structure similar to that of organic cation transporters, positively charged residues in transmembrane domains of rOAT3 are essential to recognize the anionic region of the substrate (22). Aromatic residues in the transmembrane domain of the same transporters might form a pocket to accommodate bulky side chains (23). The studies reported here scanning the whole OCTN2 cDNA confirm an essential role of transmembrane residues in substrate recognition by OCTN2. It is interesting to note that one residue identified in this study is indeed a positively charged arginine that could possibly interact with the negative charge of carnitine.

There are two important considerations. The first is that the \( V_{\text{max}} \) for carnitine transport was reduced in several single and double mutant transporters even without changes in the \( K_m \) toward carnitine (Figs. 6 and 7). These changes were not associated with abnormalities in sodium stimulation of carnitine...
transport (Fig. 7). This indicates that substitution of these residues affects not only the recognition of carnitine but also the progression of carnitine along the transmembrane domain. The second consideration is that multiple residues need to be substituted before a recognizable effect on carnitine affinity can be observed (Fig. 7). This finding is unique and indicates that multiple residues contribute to substrate binding and that probably there is redundancy of these binding sites, so that substitution of only one of them does not affect carnitine recognition but becomes evident, from a kinetic standpoint, only as a retardation of the influx of substrate inside the cell.

Acknowledgment—We thank Dr. Vadivel Ganapathy for providing the cDNAs for hOCTN1 and hOCTN2.

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