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Tryptophan 224 of the rat mitochondrial carnitine/acylcarnitine carrier is crucial for the antiport mechanism

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ABSTRACT

The mitochondrial carnitine/acylcarnitine carrier (CACT) catalyzes an antiport of carnitine and acylcarnitines and also a uniport reaction with a rate of about one tenth with respect to the antiport rate. The antiport process results from the coupling of the two uniport reactions in opposite directions. In this mechanism, the transition of the carrier from the outward open conformation to the inward open one (or vice versa) is much faster for the carrier-substrate complex than for the unbound carrier. To investigate the molecular determinants that couple the binding of the substrate with the conformational transitions, site directed mutagenesis has been employed. The antiport or the uniport reaction was followed as [^{3}H]carnitine uptake in or efflux from proteoliposomes reconstituted with the WT or Trp mutants of the rat CACT. Substitution of each the three Trp residues led to different results. Nearly no variations were observed upon substitution of W192 and/or W296 with Ala. While, substantial alteration of the transport function was observed in the mutants W224A, W224Y and W224F. Mutation of W224 led to the loss of the antiport function while the uniport function was unaltered. In these mutants impairment of the substrate affinity on the external side was also observed. The data highlights that W224 is involved in the coupling of the substrate binding with the matrix gate opening. The experimental data are in line with predictions by homology modeling of the CACT in its cytosolic (c-state) or matrix (m-state) opened conformations.

1. Introduction

The mitochondrial carriers, belonging to the SLC25 family, share peculiar structural features and molecular mechanisms [1] which have been firstly revealed by the 3D structure of the Adenine nucleotide-carrier (ANC) in a conformation opened towards the cytosol (c-state) [2]. The structures of the other members of the SLC25 family have been predicted by homology modeling, using the ANC 3D coordinates as the template (PDB code 1OKC). A bundle of six α-helix transmembrane segments, enclosing a water-filled cavity, characterizes the carrier protein structures. Repetitions of two adjacent transmembrane segments show internal similarity highlighting a tripartite structure. Short hydrophilic loops with random coiled structure, facing the inter-membrane space, connect the hydrophobic transmembrane segment repetitions while, larger hydrophilic loops with a α-helical structure, facing the matrix, connect the transmembrane segments within each couple. The substrate-binding sites are located in the central cavity at about mid of the membrane [3–8]. The recent structure of the ANC from *T. thermophila* in complex with the bongkrekic acid (PDB code 6GCI), in the matrix-opened conformation (m-state), added important information on the molecular mechanism of the transport reaction catalyzed by mitochondrial carriers. The SLC25 mitochondrial carrier family counts many members each specific for few substrates. These transporters catalyze the exchange of many metabolites from the cytosol to the mitochondrial matrix and vice-versa allowing completion of metabolic pathways which are characterized by the localization of enzymes into both compartments. To perform this task most mitochondrial carriers catalyze antiport reactions. In this transport mode, the transport of one substrate from the cytosol to the matrix requires the counter-transport of another substrate in the opposite direction. Two

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networks of six amino acid residues present at the bottom (close to the matrix) and at the top of the transporters (close to the intermembrane space) play a crucial role in the molecular mechanism of the antiport. The subsequent opening and closing of these networks together with the simultaneous rotation of the three intermembrane domains, allows substrates to enter the carrier from one side and to be translocated towards the opposite side. The occurrence of this process in the two opposite directions realizes the alternating access mechanism, which is at the basis of the antiport function. Antiport is favored with respect to uniport, since the gate opening is much faster for the carrier-substrate complex than for the empty carrier [9]. Several information on the structure/function relationships has been obtained by site-directed mutagenesis [1,4,9–15]. However, the molecular determinants responsible for coupling substrate binding with gate opening that favors the antiport, are still unknown. Some information derives from studies on the carnitine/acylcarnitine carrier (CACT) that has the peculiarity, among mitochondrial carrier, of catalyzing a measurable uniport reaction that occurs at a lower rate with respect to the antiport reaction. This special feature could allow discriminating the molecular determinants crucial for the sole antiport reaction from those important for the overall mechanism of catalysis. Previous data highlighted that an amino acid residue belonging to the matrix network, namely K35, is involved in the antiport mechanism [16]. Trp residues have been shown in bacterial antiporters, as well as in some human orthologues, to play important roles in transport mechanism both in gating and in substrate recognition [17–21]. Thus, we have now investigated the role of a Trp residue, which is conserved in the CACT sub-family and is rarely present in other mitochondrial carriers. Some crucial experiments have also been performed on the *A. nidulans* CACT which, differently from the rat transporter, contains the sole conserved Trp residue.

2. Materials and methods

2.1. Materials

Sephadex G-75 was purchased from Pharmacia, egg-yolk phospholipids (l-α-phosphatidylcholine from fresh turkey egg yolk) and Amberlite XAD-4 from Fluka, PIPES, Triton X-100, cardiolipin, N-dodecanoylsarcosine (sarcosyl), Koshland's reagent (KR) from Sigma, St. Louis, MO, L-[methyl-3H]carnitine was from Amersham. All reagents were of analytical grade.

2.2. Over-expression and isolation of the CACT proteins

The previously constructed pMW7-WT rat CACT recombinant plasmid [22] was used to introduce specific mutations (see Results) in the CACT protein as previously described [23]. The amino acid replacements were performed with complementary mutagenic primers using the overlap extension method [24] and the High Fidelity PCR System (Roche). The PCR products were purified by the QIAEX II Gel Extraction Kit (QIAGEN La Jolla), digested with *Nde*I and *Hin*dIII (restriction sites added at the 5′ end of forward and reverse primers, respectively) and ligated into the pMW7 vector that was previously digested with the same restriction enzymes. Sequencing and transformation of the selected clones into *Escherichia coli* C0214, bacterial over-expression, isolation of the inclusion body fraction, solubilization and purification of the wild-type CACT and mutant CACT proteins were performed as described previously [23,25].

In order to over-express the fungus *Aspergillus nidulans* carnitine/acylcarnitine carrier (AcuH) protein, the CACT cDNA obtained as previously described [26] was amplified using the forward primer tatatcatatgtccgccgccgaatcgccc and the reverse primer gaacggatccttagtcgaaaagcttgttcatga, containing the *Nde* I and BamHI restriction sites, respectively, for cloning into the pMW7 vector. The purified cDNA was cloned in pMW7 for transforming *E. coli* C0214 and over-expressing the protein. Isolation, solubilization and purification of the wild-type AcuH were performed as described above for the rat CACT [23,25]. To introduce specific mutations in the AcuH proteins the same procedure used for the rat CACT was used [23,24].

2.3. Reconstitution of wild-type (WT) and mutant proteins in liposomes

The recombinant proteins (60 μg protein) were reconstituted into liposomes as described previously [7,23,25]. The concentration of intraliposomal carnitine was 15 mM except when differently specified in the legends to figures. The orientation of the reconstituted protein was the same as in the native membrane as previously described [16].

2.4. Transport measurements

In order to remove the external substrate for antiport or uniport assay, 550 μ1 proteoliposomes obtained as in Section 2.3, were passed through a Sephadex G-75 column (0.7 cm × 15 cm), equilibrated with 10 mM PIPES pH 7.0 and 90 mM NaCl. The eluted proteoliposomes (600 μl), distributed in reaction vessels (100 μl), were used for transport measurements using the inhibitor-stop method [27]. Transport was performed at 25 °C, started by adding 0.1 mM [^{3}H]carnitine (except that in kinetic experiments in which different [^{3}H]carnitine concentrations were used) to proteoliposomes and terminated by the addition of 1.5 mM NEM (N-ethylmaleimide), a specific inhibitor of CACT. In control samples, the inhibitor was added together with the radiolabeled substrate at time zero. After stopping the transport reaction, the radioactivity not taken up by the proteoliposomes was removed by passing 100 μl of each sample through a Sephadex G-75 column (0.6 cm × 8 cm). The proteoliposomes were eluted with 1.2 ml 50 mM NaC1 and collected in 4 ml scintillation mixture, vortexed and counted. The experimental values were corrected by subtracting control values. For kinetic experiments, initial transport rate was derived from the initial linear part of the time course of the isotope equilibration kinetics. For the purpose of performing efflux experiments, proteoliposomes were filled with the indicated concentration of [^{3}H]carnitine (intraliposomal carnitine) before starting efflux transport in uniport mode or antiport mode [16]. NEM (1.5 mM) was added for stopping the transport reaction at various times. The decrease in radioactivity inside the proteoliposomes was measured and expressed as percent of the radioactivity present at time zero [16,27].

3. Results

3.1. Location of the Trp residues in the CACT homology structural model

The rat CACT contains three Trp residues, namely W192, W224 and W296. W224 is conserved along with the different species of the CACT subfamily while the others are present only in the vertebrate CACTs (Fig. 1). The predicted positions of the Trp residues are depicted in the homology structural model of CACT by licorice representation (Fig. 2). W224 is located in the central aqueous cavity of the carrier, in the vicinity of other residues that constitute the matrix gate of the transporter [8]. W192 is located towards the cytosolic side of the transporter (Fig. 2). W296 is also located in the cytosolic side, but is not depicted in

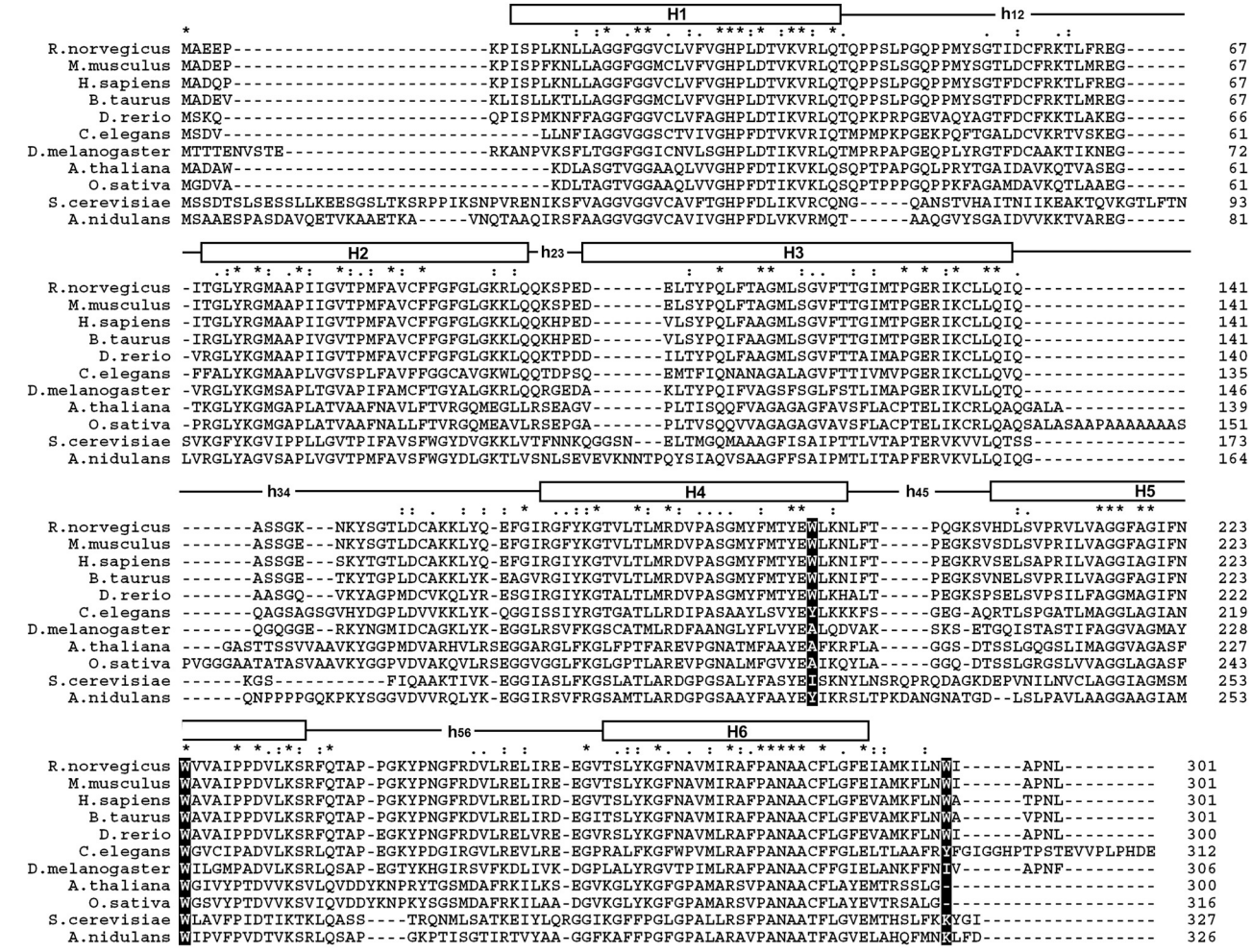

Fig. 1. Alignment of proteins of the mitochondrial carnitine carrier subfamily. CACT proteins (NP446417 from *Rattus norvegicus*, NP000378 from *Homo sapiens*, NP568670 from *Arabidopsis thaliana*, NP014743 from *Saccharomyces cerevisiae*, AJ011563 from *Aspergillus nidulans*, NP065266 from *Mus musculus*, XP001253588 from *Bos taurus*, NP957153 from *Danio rerio*, NP501223 from *Caenorhabditis elegans*, NP477221 from *Drosophila melanogaster*, and NP001065471 from *Oryza sativa*) were aligned using the Clustal Omega software. Identities are indicated by asterisks and conservative or highly conservative substitutions are indicated by dots or colons, respectively. The three Trp residues 192, 224 and 296 are highlighted by dark background.

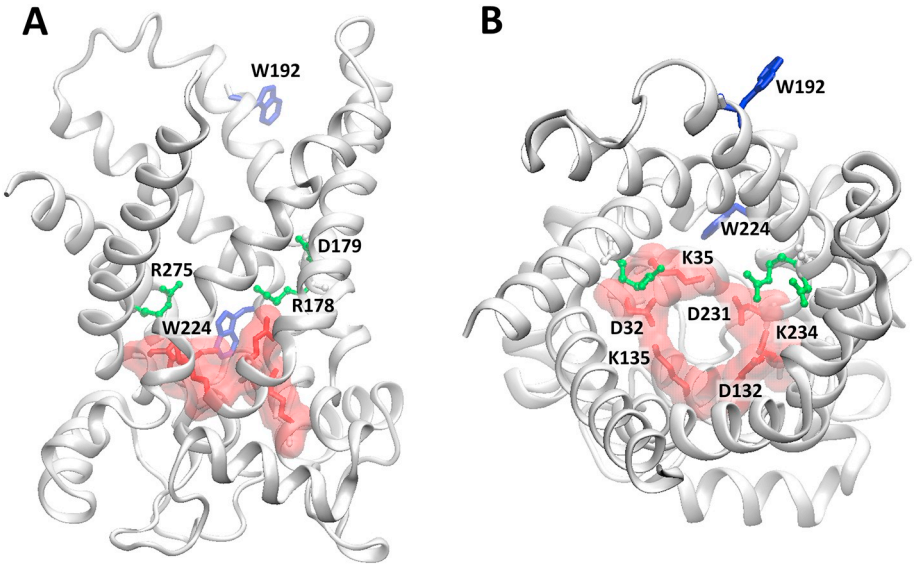

Fig. 2. Location of the CACT Trp residues in the homology structural model.
The homology model has been obtained by Swiss model portal using as templates the 1OKC PDB and represented by the molecular visualization program VMD. A) Lateral view of CACT in c-state depicted as a ribbon diagram. Residues of the matrix gate are highlighted in red with a transparent space filed conformation, the residues of the central binding site are depicted in green with a ball and stick diagram while Tryptophans are highlighted in blue as sticks graphic representation. B) Top view of the same CACT structure.

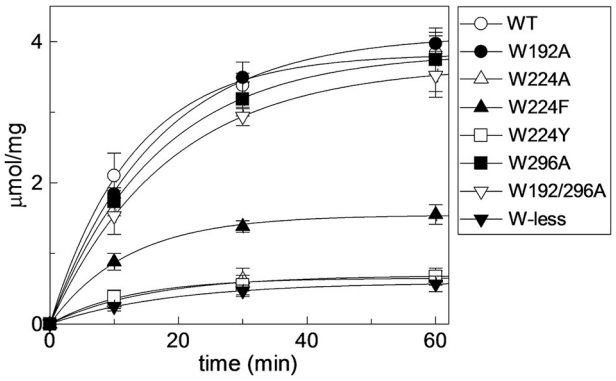

Fig. 3. Time course of [^{3}H]-carnitine uptake in liposomes reconstituted with recombinant WT and Trp mutant proteins of CAC. Transport was started by the addition of 0.1 mM [^{3}H]-carnitine to proteoliposomes containing 15 mM carnitine and stopped at the indicated times. The plot shows the time course curves of WT and mutant CACT as reported in the figure legend. The data represent means ± SD of at least three independent experiments.

Table 1
Kinetic analysis of WT and mutant CACTs. Kinetic analysis was performed as described in Materials and methods. Initial transport rates were measured at various substrate concentrations as shown for Fig. 4 B and interpolated in the Lineweaver-Burk equation for obtaining the Km and Vmax values. The reported data are means ± S.D. from three independent experiments.

Protein	External Km (mM)	Vmax (µmol/min/mg)	Vmax/Km (ml/min/mg)
WT	0.40 ± 0.10	1.3 ± 0.7	3.3
W192A	0.66 ± 0.014	1.6 ± 0.071	2.4
W224A	3.7 ± 1.5	0.42 ± 0.19	0.11
W224F	3.2 ± 0.86	0.38 ± 0.068	0.12
W224Y	3.3 ± 1.0	0.31 ± 0.12	0.10
W296A	0.31 ± 0.0071	0.61 ± 0.12	2.0
W192/296A	0.47 ± 0.080	1.4 ± 0.7	3.0
W-less	4.4 ± 0.52	0.26 ± 0.16	0.060

the CACT structural model, since this structure derives from that of the ADP/ATP carrier that lacks the last C-terminal amino acids.

A role of W224 in substrate binding and translocation was expected, since this residue is located in the core of the protein, close to the matrix gate and to the substrate binding site. The Trp residues were substituted by Ala, generating the single mutants W192A, W224A, and W296A. Since W224A was the sole mutant showing impaired function (see below), additional mutants were constructed to better understand the role of W224, i.e., the double mutant W192A/296A, and the triple mutant W-less. W224 was also substituted by the conservative amino acids Phe and Tyr, generating the W224F and W224Y mutants. The recombinant mutant proteins were over-expressed in *E. coli* and reconstituted in liposomes as described in Material and methods section [23]. The amount of each mutant protein incorporated into proteoliposomes was very similar to that of the WT (not shown) and ranged between 25 and 35% of the recombinant protein added for reconstitution, as found for many other previously constructed mutants of CACT [8,16,23].

3.2. Transport activity of the Trp mutants of CACT

The transport activities of WT and Trp mutants were measured as a function of time. Fig. 3 shows that W192A, W296A and the double mutant W192/296A (containing only W224) exhibited transport activities comparable to that of WT; whereas the single mutants W224A, W224Y and the W-less showed much lower transport activities, with transport at 60 min of about 15% of WT. The mutant W224F showed a transport activity slightly higher than the other mutants lacking W224. The low activity of the mutants lacking W224 suggests a crucial role for this residue in transport function. To gain further insights into the relationships among W224 and the transport mechanism, the analysis of the kinetic parameters of the mutant CACTs were performed (Table 1) in comparison to those of the WT. Km values more than eight times that of WT were measured for the mutants lacking W224. While, the mutants W192A, W296A and W192/296A displayed a Km value similar to that of the WT. The Vmax was also impaired in the W224A, W-less, W224F and W224Y mutants, while, the W192A, W296A and the double W192/296A mutants showed Vmax similar or not very different from that of the WT (Table 1). The catalytic efficiencies of the mutants were calculated and compared with that of the WT. The impairments of catalytic efficiencies reflected the impairments in Km and Vmax. The strong increases in Km (decreased affinity) of the W224 mutants indicates that this residue could be crucial in the interaction with the substrate in some steps of the transport process.

3.3. Inhibition of the WT and CACT mutants by the Koshland's reagent

Chemical modification of protein can give additional information for identifying amino acid residues involved in the binding of the substrate [8,28,29]. Koshland's reagent (KR) specifically reacts with tryptophan residues and, then, could be employed for proofing the involvement of Trp residues in the interaction with the substrate [30,31]. Inhibition by this reagent was tested on the transport activity of WT CACT and on some representative mutants. Both the WT and the W192/296A mutants (Fig. 4A) displayed strong inhibition by the reagent. From the dose-dependence analysis, half inhibition constants (IC_{50}) were derived. The calculated values, 2.7 ± 0.35 mM for the WT and 2.9 ± 0.25 mM for the W192/296A, are very similar and confirm that the two proteins share the same response to the modification of Trp residue(s). As expected, the mutant W-less was insensitive to the reagent. The mutant W224A was also insensitive as the W-less, indicating that the sole modification of W224 is responsible for the activity loss caused by the reagent. However, it cannot be excluded that W192 and/or W296 could be modified by the reagent, but, in this case, the reaction has no consequences on the transport function, indicating that the residues are not critical for function and are located in protein moieties not involved in the transport path, according to the structure (Fig. 2). Very interestingly, the Koshland's reagent did not have any effect on WT and on the W292/296A double mutant when the uniport activity was measured instead of antiport. This data suggests that the sole antiport function is sensitive to the chemical modification of W224. The single mutants W192A and W296A showed the same behavior as the WT (not shown) according to the general features of these mutants (see also Table 1). To investigate the relationships between W224 and the substrate binding site, inhibition kinetics were performed in the presence or absence of the Koshland's reagent and the data was analyzed by a double reciprocal plot, as shown in Fig. 4B. The data points interpolate straight lines intersecting on the X axis, indicating a non-competitive inhibition. The half saturation constants (Ki) calculated from the graph were 8.5 ± 1.3 or 5.9 ± 1.0 mM for the WT or the mutant W192/296A, respectively. This pattern suggests, apparently, that the inhibitor binding site could be far from the substrate binding site. However, in the case of covalent inhibition, this type of pattern can occur even if the reagent target is close to the substrate binding site [32]. Thus, a protection analysis was performed, that in such cases may reveal the interaction of the inhibitor and the substrate with the same (or vicinal) sites. To investigate the possible protective effect of carnitine on the binding of the Koshland's reagent to the sole W224, the proteoliposomes reconstituted with the double mutant W192/296A (containing the sole W224) were incubated with the inhibitor in the presence or absence of carnitine. Koshland's reagent concentration was kept close to its Ki. As shown in Fig. 4C, the mutant exhibited substantial protection by the substrate. Indeed, the 70% inhibition of the control, without carnitine pre-incubation, decreased to about 50% and

38% upon pre-incubation with 0.5 or 10 mM carnitine, respectively. This data correlates well with the vicinity of the W224 to the residues of the matrix network and to the residues of the substrate binding site (Fig. 2).

In the basis of the described data, it seems that the substitution of W224 impairs the coupling of the substrate binding with the matrix network opening, abolishing the antiport function. This hypothesis correlates well with the data of Fig. 3, in which the transport activity of the mutant W224 is about 10% that of WT indicating that the impaired function could be only due to the absence of stimulation by the counter substrate with the sole remaining uniport function.

3.4. Loss of antiport function of the W224 CACT mutants

To gain further insights into the impairment of the antiport function, the stimulation by the countersubstrate was tested by measuring the transport activity as [^{3}H]carnitine transport in the presence of increasing internal substrate concentrations (Fig. 5). As expected for the WT, transport increased by increasing the intraliposomal carnitine concentration according to the antiport mode. While, in the case of the W224A mutant, no variations of [^{3}H]carnitine uptake were measured,

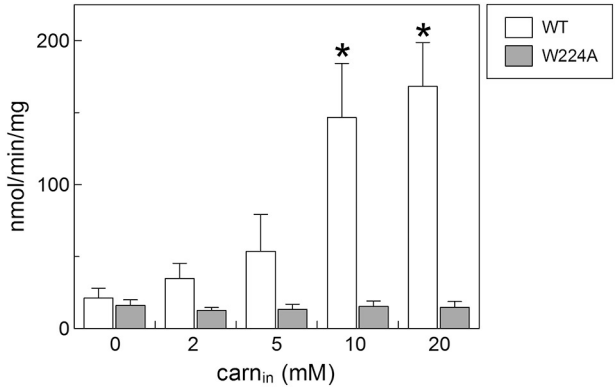

Fig. 5. Stimulation of carnitine transport by internal countersubstrate. Transport was started by the addition of 0.1 mM [^{3}H]-carnitine to proteoliposomes containing the indicated carnitine concentrations and stopped at 10 min. The data related to the WT or W224A CACT proteins are shown as white or gray bars, respectively. The data represent means ± SD of three independent experiments. (*), Significantly different from the control (without intraliposomal carnitine) for $p < 0.05$, as calculated from Student's *t*-test analysis.

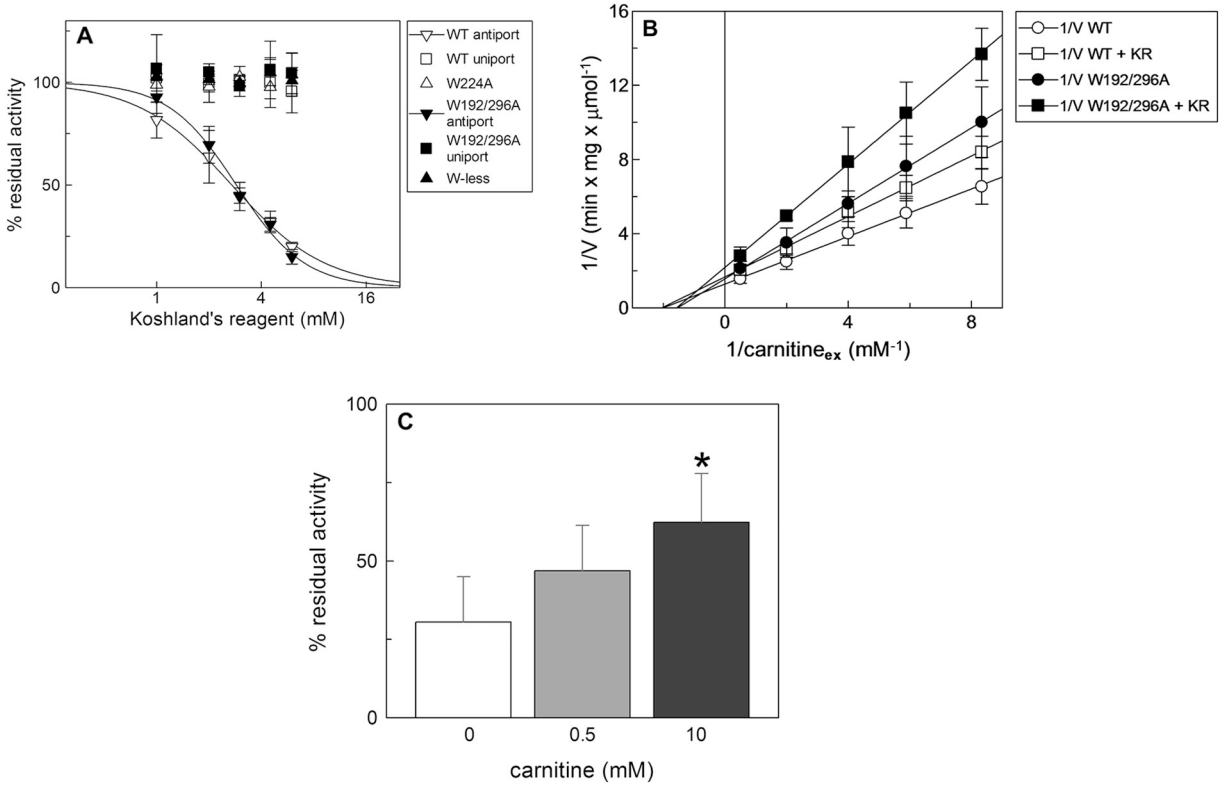

Fig. 4. Inhibition of the CACT by Koshland's reagent. (A), Dose-response curve for the inhibition of the reconstituted recombinant WT protein and Trp mutants of CACT. The antiport rate was measured adding 0.1 mM [^{3}H]carnitine to proteoliposomes containing 15 mM internal carnitine. The uniport rate was measured adding the labeled carnitine to proteoliposomes without internal substrate. KR, at the indicated concentrations, was added 2 min before the transport assay. Percent of residual activities are reported. The values are means ± S.D. from three independent experiments. (B), Kinetic analysis of the effect of Koshland's reagent on the WT and W192/296A proteins. The antiport rate was measured adding [^{3}H]carnitine at the indicated concentrations to proteoliposomes containing 15 mM internal carnitine, in the absence (○, □) or in the presence of 3.0 mM KR added together with the labeled substrate (●, ■). Experimental data are plotted according to Lineweaver-Burk as reciprocal transport rate vs reciprocal carnitine concentrations. Reported values are means ± S.D. from three independent experiments. (C), substrate protection of the W192/296A CACT inhibition by KR. Carnitine at the indicated concentrations (0.5 and 10 mM) was added to proteoliposomes passed through a Sephadex G-75 column and incubated for 5 min with KR 3.0 mM. Then, the unreacted compound was removed by passing the proteoliposomes through a second Sephadex G-75 column. Transport was started by adding 0.1 mM [^{3}H]carnitine to the proteoliposomes and stopped after 30 min. Percent residual activity is reported with respect to the control without the addition of the inhibitor. Reported values were the means ± SD from three different experiments. (*), Significantly different from the control (without added carnitine) for $p < 0.05$, as calculated from Student's *t*-test analysis.

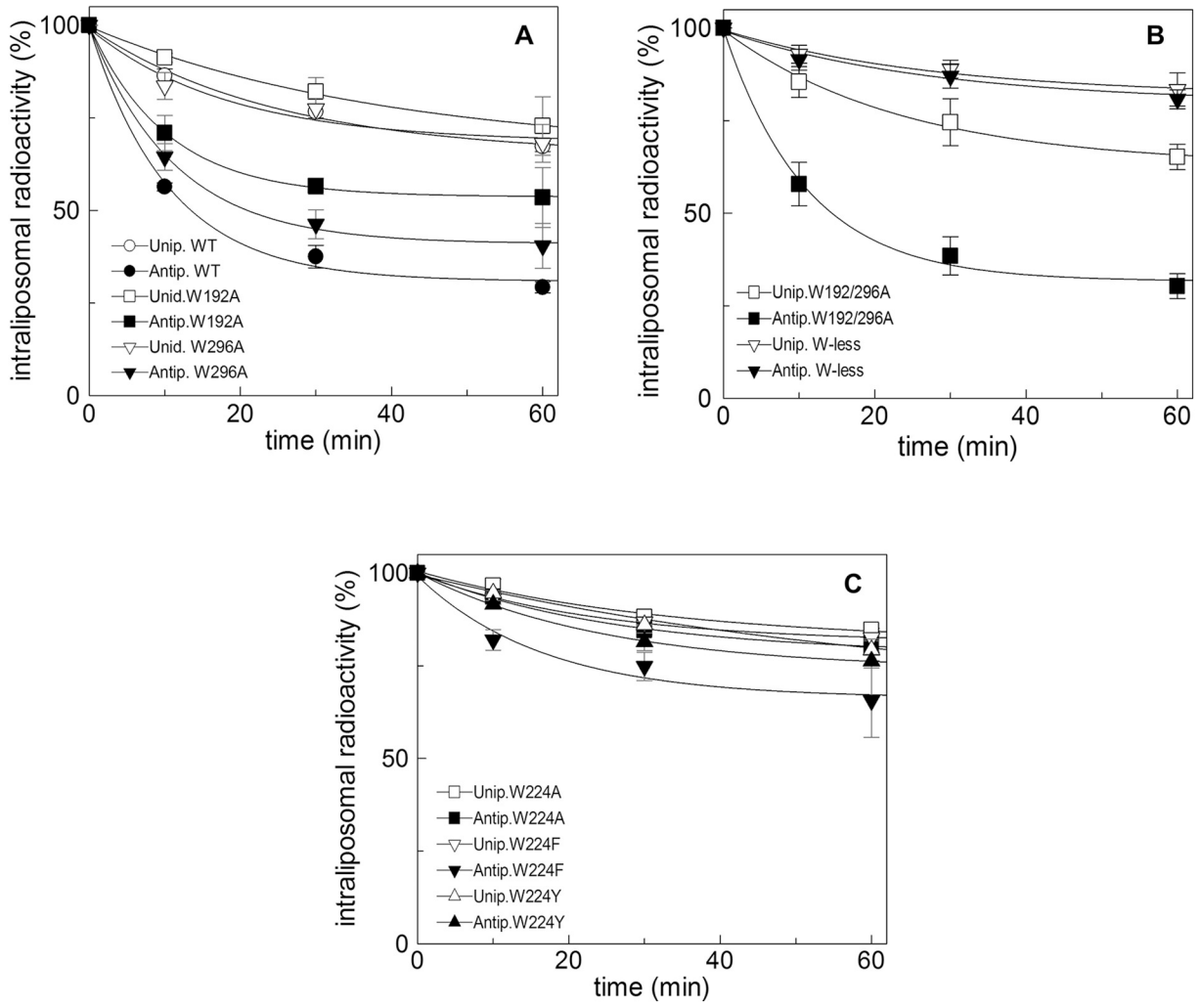

Fig. 6. Antiport and uniport function of WT and mutant CACTs.
To measure the transport activity of CACT using the efflux approach, proteoliposomes reconstituted with WT or W mutants, were preloaded with 15 mM [^{3}H] carnitine, then the efflux of labeled carnitine was monitored along time. The residual intraliposomal radioactivity was measured at the indicated times in the presence of 5 mM external carnitine (antiport) or buffer alone (uniport). The proteins (WT or mutants tested) in A, B and C are indicated in the figures. The values are means ± SD from three experiments.

i.e., no stimulation by the countersubstrate could be detected, confirming that this mutant catalyzes only the residual uniport function.

For a final proof of this hypothesis, the efflux procedure was employed which is the most reliable to discriminate between antiport and uniport function (Fig. 6). To this aim, [^{3}H]carnitine efflux from preloaded proteoliposomes was measured in the absence (uniport) or presence (antiport) of the external substrate. The WT proteoliposomes behaved as expected, i.e., antiport was much more efficient than uniport. Similarly, the mutants in which one of the two non-critical Trp residues were substituted, showed an antiport function much more efficient than the uniport. Again, a similar result was obtained with the double mutant W192/296A (Fig. 6B). On the contrary, the W224A and the W-less did not show stimulation by external substrate, demonstrating that the antiport function was impaired. Slight stimulation of the antiport by the addition of the external substrate was only observed with the W224F (Fig. 6 C). The efflux procedure was also used to measure the internal Km for W224 in comparison with the WT. Similar values were obtained, i.e., 7.3 ± 2.8 mM or 6.7 ± 3.0 mM (from 3 independent experiments) for the WT and the W224A, respectively, suggesting that W224 is not involved in substrate binding from the internal side.

To further support the specific role of W224 in the antiport mechanism, the same mutation was performed on the *A. nidulans* CACT, that is quite different from the human protein, but has the sole W224 homologous residue, W254. The antiport and uniport reactions were measured in proteoliposomes by the efflux procedure (Fig. 7). Also in this case, the antiport function virtually disappeared in the mutant W254A, confirming that this residue is crucial for the antiport mechanism.

4. Discussion

The molecular determinants of the transport mechanism of SLC members are little known. More information is available for bacterial

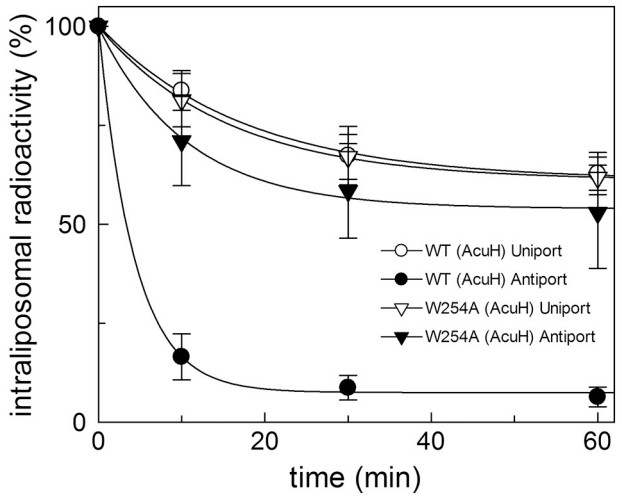

Fig. 7. Antiport and uniport function of WT and W245A mutant *A. nidulans* CACT.

To measure the transport activity of *A. nidulans* CACT using the efflux approach, proteoliposomes reconstituted with WT or W245A mutant, were preloaded with 15 mM [3H]carnitine, then the efflux of labeled carnitine was monitored along time. The residual intraliposomal radioactivity was measured at the indicated times in the presence of 5 mM of external carnitine (antiport) or buffer alone (uniport). The values are means ± SD from three experiments.

[17,20] than for eukaryotic transporters. Concerning the mitochondrial carriers, these transport proteins can be assimilated to single binding center gated pores, as some bacterial transporters. However, mitochondrial carriers are unique with respect to their structure and, hence, mechanism since these proteins are constituted by a bundle of six transmembrane segments arranged in three intramembrane domains which rotate to allow conformational changes for transport reaction [11,33]. The structures of the bovine and yeast ADP/ATP carriers were firstly available in the c-state conformation opened towards the cytosolic side [2,34]. The structure of the ADP/ATP carrier from *T. thermophila* is now available in the m-state, as well [9]. This last structure led to significant advancements in defining the conformational changes required for substrate transfer from one side of the membrane to the other. This information together with in-silico analysis and site-directed mutagenesis studies, indicate that substrate binding induces the conformational changes required for transport [9]. The antiport mechanism is determined by the coupling of substrate binding with gate opening. Indeed, the rate of transition of the carrier from an outward open conformation (c-state) to the inward open one (m-state) or vice versa, is forbidden or much slower for the unbound carrier than for the carrier-substrate complex [33].

In this work, we provide further insights into the mechanism of coupling of substrate binding with the opening of the matrix gate of the CACT, which is at the basis of the antiport mechanism. This transporter is suitable to gain insights into the molecular mechanism of the antiport since it is among the few transporters that, besides antiport, also catalyzes a uniport reaction with a lower rate than the antiport. This peculiarity has been proposed since the 80s in studies performed with intact mitochondria [35] and then confirmed by detailed studies performed with both the native protein from rat liver and the recombinant rat and human proteins [16,36]. The co-existence of the two modes allows discriminating mutations, which impair the overall transport cycle from those, which specifically impair antiport. Using this strategy, we identified an amino acid residue, namely K35, whose substitution caused a reduction of transport stimulation by counter-substrate, impairing the antiport function. Those findings suggested that K35 should be involved in the binding of the carboxyl moiety of carnitine favoring the gate opening. However, at least one companion amino acid residue should then be involved in the binding of the ammonium moiety of carnitine that, together with K35, facilitates the opening of the transporter towards the matrix. In the present work, the second, most crucial residue has been identified, namely W224. Indeed, the substitution of W224 completely abolishes the antiport, leaving the sole uniport function. The protein lacking W224 is thus converted into a uniporter with a transport activity equal to the unidirectional activity of the WT. Indeed, the effect of mutation of W224 on antiport impairment is much more evident than in the case of substitution of K35 [16]. Moreover, the Koshland's reagent that reacts with Trp residues, affect only the antiport function, but not the uniport, confirming that W224 is crucial for antiport. Altogether, the results indicate that W224 is the major residue involved in coupling and it undergoes to a cation-π bond with the ammonium moiety of carnitine. It is well known than cation-π is the strongest among the non-covalent interactions [37]. Indeed, betaine, which shares a quaternary ammonium moiety with carnitine, binds to Trp residues in the bacterial Glycine Betaine Transporter [37,38]. In CACT, the interaction of carnitine with the two residues W224 and K35, the second of which is in the matrix gate [16], induces a fast opening of the gate. In the absence of the substrate, the gate opening is much slower. The distance of W224 from K35 (3 Å) corresponds to the distance between the carboxylic and the ammonium groups of carnitine (Fig. 8A and B). Indeed, the substitution of W224 impairs this catalytic step and the opening process is slowed down, matching the rate of transition of the unbound carrier. The substitution overlaps the effect of chemical modification [30] of the W224, leading to the same results. The coupling of substrate binding with gate opening occurs when the substrate enters the carrier from the external side (Fig. 8A and B). This correlates well with the increase in external Km of the proteins lacking W224 with respect to the WT. While, W224 is not important for the substrate binding and transport from inside to outside, i.e., when the matrix gate is opened. In this state the distance between W224 and K35 is greater than in the c-state and, hence, the two residues are not suitable for binding of the internal substrate (Fig. 8C and D). This again correlates with the lack of changes in the internal Km upon substitution of the W224. The experimental data correlates very well with the change of the reciprocal position of the two residues in the structures of the CACT in the c-state and in the m-state (Fig. 8) obtained by homology modeling on the basis of the two structures of the ADP/ATP carrier, respectively [2,9]. The data show that the antiport is impaired in both the directions since the lack of one of the fast steps (from outside to inside) limits the overall rate of conformational transitions. The binding is only impaired from the external (higher Km) since only the residue involved in binding from the external side is substituted. Hence, the residue(s) involved in binding and gate opening from the internal side, if present, should be different and probably located in the vicinity of the cytosolic gate residues [5].

The data are also in line with the described interaction of the CACT with omeprazole. Interestingly, W224, as well as K35 are among the residues interacting with omeprazole molecules docked into the CACT structure. Given that omeprazole is not transported, the inhibitor may bind the two residues involved in carrier opening without inducing conformational changes, but blocking the carrier in a non-functional conformation. The occurrence of many other interactions with omeprazole prevents the carrier opening [39].

W224 is present also in the Ornithine/Citrulline carrier isoforms (SLC25A15/A2) and in the mitochondrial transporter for basic amino acids (SLC25A29) [40]. Indeed, the substrates of these transporters show some similarity with carnitine, i.e., a carboxylic as well as a charged amino group at a distance of 3–4 Å. Interestingly, the previously reported mutation of W224 with Ala in the ORNT1 (SLC25A15), causes a strong reduction of antiport of Ornithine and Arginine, while the very slow unidirectional transport (with sodium inside the proteoliposomes) remains about the same of the WT protein [41]. Moreover, also the SLC25A29 shows a uniport function with a lower efficiency with respect to the antiport function similarly to the CACT [40]. The lack of this Trp residue in other carriers indicates that the highlighted

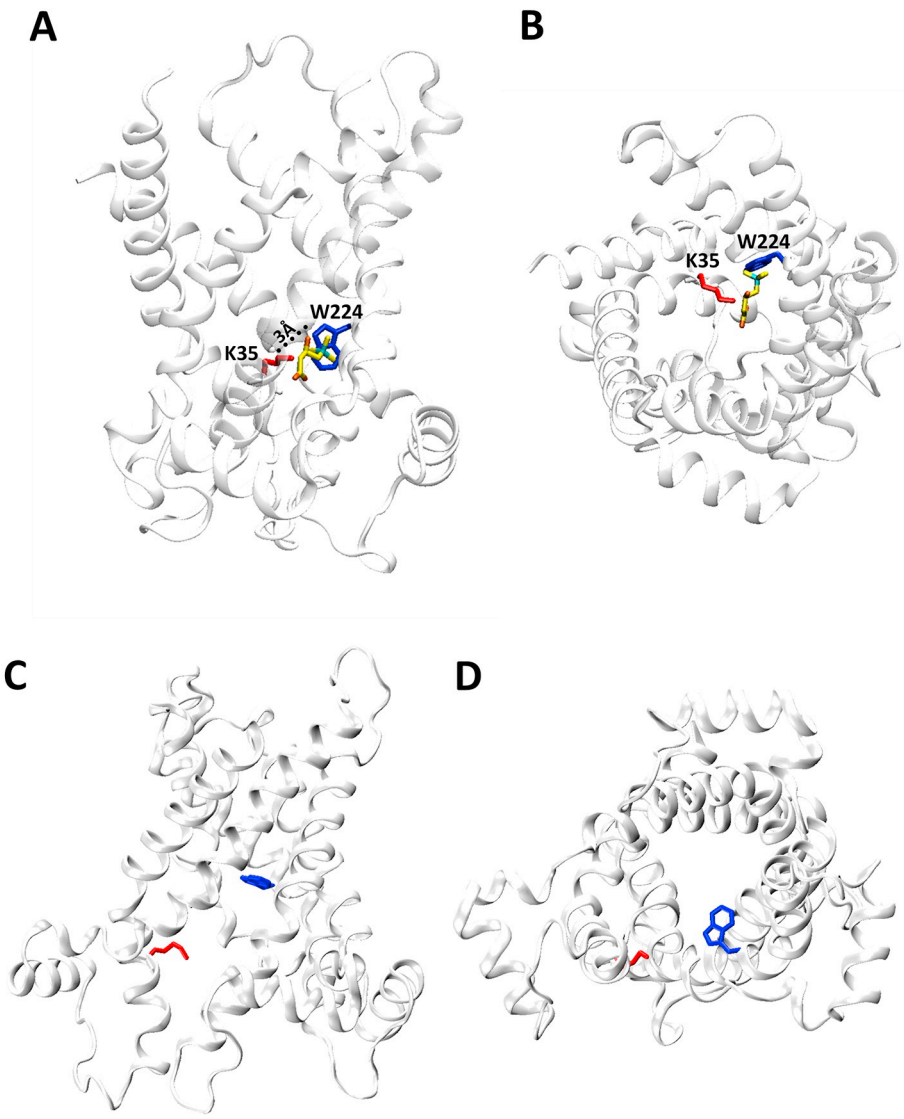

Fig. 8. Amino acid residues of CACT involved in the coupling of substrate binding with gate opening. The homology models of CACT in c-state and m-state have been obtained by Swiss model portal using as templates the 1OKC or 6GCI PDB, respectively and represented by the molecular visualization program VMD, as indicated in the Fig. 2 legend. Carnitine docking analysis has been performed using ArgusLab software. A) Lateral view of CACT structure in c-state with bound carnitine was highlighted as a ribbon diagram. Trp 224 and K35 were in blue and red respectively. Carnitine was depicted with a licorice conformation. B) Top view of the same CACT structure. C) Lateral view of CACT structure in m-state highlighted as a ribbon diagram. Trp 224 and K35 were in blue and red respectively. D) Bottom view of the same CACT structure.

mechanism might be typical of transporters handling substrates with a positively charged functional group. Carriers handling anions must have different mechanisms or different type of amino acid residues involved in similar mechanisms.

Transparency document

The Transparency document associated with this article can be found, in online version.

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