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Identification of Amino Acid Residues Underlying the Antiport Mechanism of the Mitochondrial Carnitine/Acylcarnitine Carrier by Site-Directed Mutagenesis and Chemical Labeling

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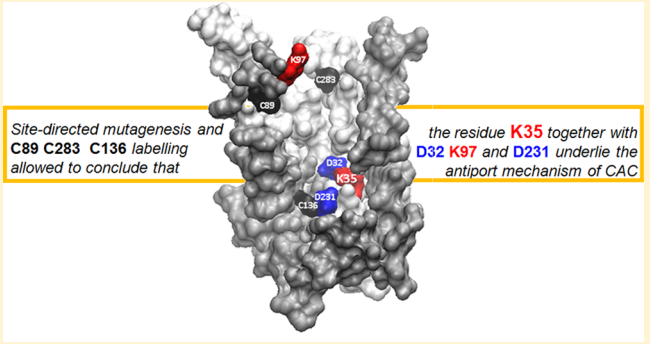
ABSTRACT: The mitochondrial carnitine/acylcarnitine carrier catalyzes the transport of carnitine and acylcarnitines by antiport as well as by uniport with a rate slower than the rate of antiport. The mechanism of antiport resulting from coupling of two opposed uniport reactions was investigated by site-directed mutagenesis. The transport reaction was followed as [^{3}H]carnitine uptake in or efflux from proteoliposomes reconstituted with the wild type or mutants, in the presence or absence of a countersubstrate. The ratio between the antiport and uniport rates for the wild type was 3.0 or 2.5, using the uptake or efflux procedure, respectively. This ratio did not vary substantially in mutants H29A, K35R, G121A, E132A, K135A, R178A, D179E, E191A, K194A, K234A, and E288A. A ratio of 1.0 was measured for mutant K35A, indicating a loss of antiport function by this mutant. Ratios of >1.0 but significantly lower than that of the wild type were measured for mutants D32A, K97A, and D231A, indicating the involvement of these residues in the antiport mechanism. To investigate the role of the countersubstrate in the conformational changes underlying the transport reaction, the m-state of the transporter (opened toward the matrix side) was specifically labeled with *N*-ethylmaleimide while the c-state of the carrier (opened toward the cytosolic side) was labeled with fluorescein maleimide. The labeling results indicated that the addition of an external substrate, on one hand, reduced the amount of protein in the m-state and, on the other, increased the protein fraction in the c-state. This substrate-induced conformational change was abolished in the protein lacking K35, pointing to the role of this residue as a sensor in the mechanism of the antiport reaction.



Mitochondrial carriers constitute a large family of proteins that catalyze the transport of metabolites through the inner mitochondrial membrane, allowing connection of cytosolic and mitochondrial metabolisms. These transporters, which do not have bacterial homologues, display a unique structural fold highlighted after the resolution, by X-ray crystallography, of the ADP/ATP carrier structure.[1] Using this template, several structures of other carriers have been predicted by homology modeling. Very recently, the structures of two isoforms of the yeast ADP/ATP carrier have been obtained by X-ray crystallography.[2] However, several aspects concerning the molecular mechanisms of mitochondrial carriers are still not clear, because the structures have been obtained only in the cytosolic open (c-state) conformations in complex with the inhibitor carboxyatractiloside. A still unsolved important question concerning these transporters is the mechanism of coupling of the countertransport of specific substrates in the antiport reactions that are common to most of the members of the mitochondrial carrier family.[3] Among these, the carnitine/acylcarnitine carrier (CAC), which plays an essential role in the β-oxidation of fatty acids,[4−6] has been well characterized, and its structural model has been validated by many experimental data obtained by site-directed mutagenesis, chemical labeling, and functional analysis in proteoliposomes.[7−11] The CAC possesses all the features of the mitochondrial carrier protein family: a primary structure partitioned in three homologous segments of ∼100 amino acids, each containing the signature motif PX[DE]XX[RK]; and the presence of six hydrophobic transmembrane segments (H1−H6) connected by five hydrophilic loops, two of which are smaller and exposed toward the cytosolic side (h_{23} and h_{45}) and three toward the matrix side (h_{12}, h_{34}, and h_{56}) of the protein.[12] The transporter that has been mainly characterized in reconstituted liposomes is functionally asymmetrical and

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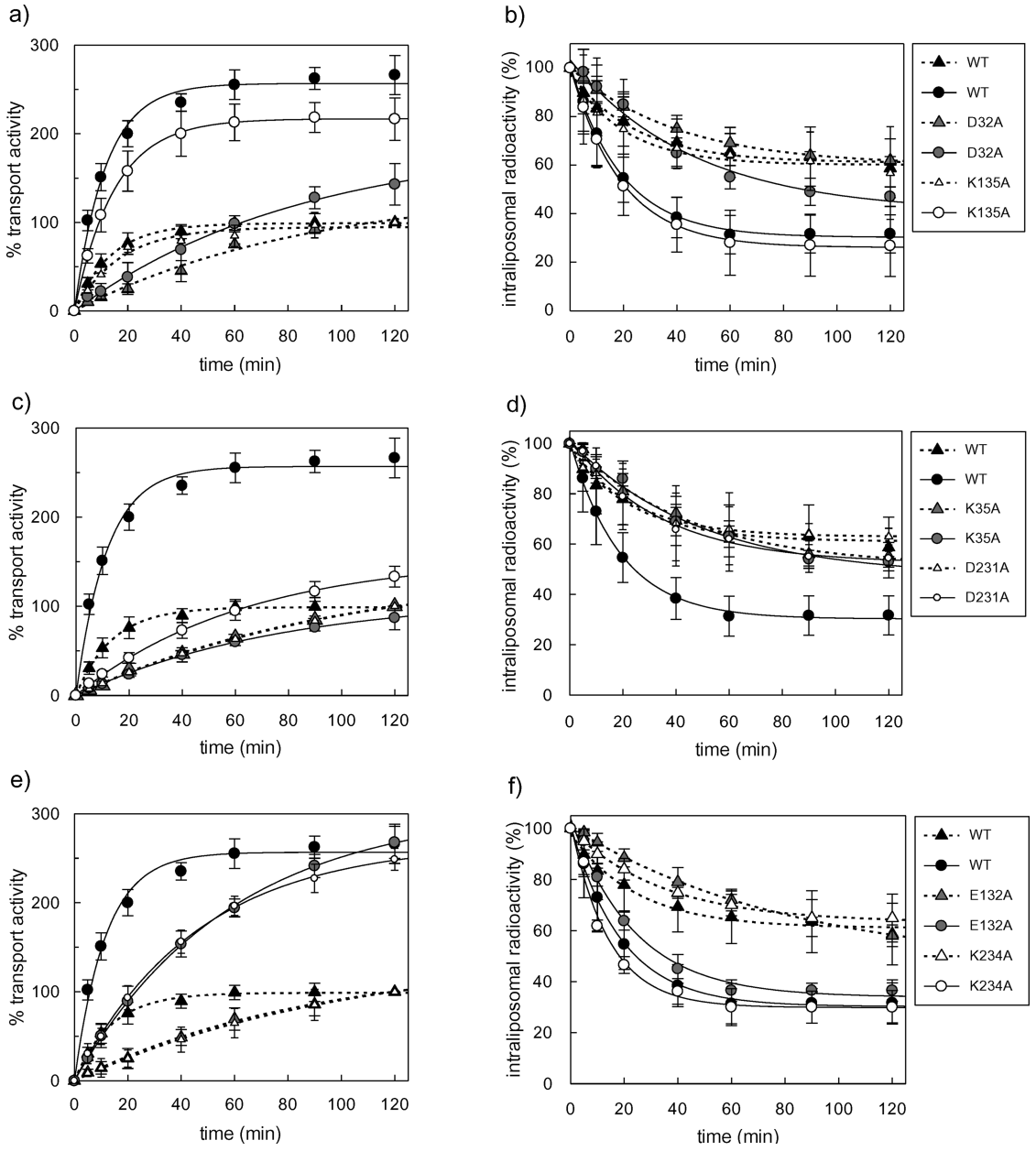

Figure 1. Antiport and uniport activity of WT and mutant CAC proteins of the matrix gate charged residues. (a, c, and e) Uptake was started by the addition of 0.1 mM [^{3}H]carnitine to proteoliposomes with (antiport) or without (uniport, dotted lines) 5 mM carnitine. (b, d, and f) Efflux of 5 mM [^{3}H]carnitine from prelabeled proteoliposomes measured as described in Experimental Procedures by adding buffer alone (uniport, dotted lines) or 2 mM unlabeled external carnitine (antiport). Data are reported as a percentage of the transport at equilibrium in the uniport mode of the WT. The data represent means ± SD of at least four independent experiments.

oriented in the proteoliposomal membrane as in mitochondria. The recombinant rat[13,14] and human[15,16] CAC proteins exhibit overlapping functional properties that are virtually identical to those of the native rat protein. In analogy with the ADP/ATP carrier, the CAC contains two salt bridge networks, one on the matrix side and the other on the cytosolic side. The matrix network consists of six conserved charged residues (D32, K35, E132, K135, D231, and K234), closing the central cavity of the transporter toward the matrix side. This state corresponds to the c-conformation in which the ADP/ATP carrier has been crystallized. A second cytosolic network, hypothesized for all the carriers on the basis of computational analysis,[17] contains in the CAC only four charged residues (K97, E191, K194, and E288) and two uncharged residues (G94 and M291). These structural features have been confirmed by site-directed mutagenesis studies that, moreover, demonstrated that in the matrix network, K35 and E132 play a major role in opening and closing the gate allowing substrate translocation.[16] The CAC catalyzes mainly carnitine/carnitine homologous and carnitine/acylcarnitine heterologous antiport reactions. In a manner different from that of most of the mitochondrial carriers that catalyze obligatory antiport reactions, the CAC also catalyzes a slower carnitine uniport that can be revealed in the absence of a countersubstrate.[15,18] For these reasons, the CAC represents a suitable model for gaining insight into the molecular mechanism of coupling that is responsible for the antiport function mode of mitochondrial carriers.

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EXPERIMENTAL PROCEDURES

Production of CAC WT and Mutant Proteins and Insertion into Liposomes. The previously obtained cDNA of WT and the mutant CAC cloned in pMW7[16] were used to express and purify the CAC proteins as described in ref 7. A reconstitution mixture composed of 60 μg of purified protein, 1% Triton X-100, 10 mg of phospholipids in the form of sonicated liposomes, 10 mM Pipes (pH 7.0), and carnitine at the indicated concentration in a final volume of 700 μL was prepared. This mixture was then passed 15 times through the same Amberlite column (0.5 cm diameter, filled with 0.5 g of resin). By this procedure, the recombinant proteins were inserted into the liposomal membranes to obtain functional proteoliposomes in which the protein is oriented as in the mitochondrial inner membrane.[7]

Assay of Transport. The substrate present in the external proteoliposomal compartment was removed by Sephadex G-75 gel filtration chromatography as described in ref 19. After this procedure, transport was started at 25 °C by adding 0.1 mM [^{3}H]carnitine to aliquots of 100 μL of proteoliposomes containing 5 or 0 mM carnitine for measuring antiport or uniport activity, respectively. Then, the reaction was stopped by the addition of the inhibitor NEM at a concentration of 1.5 mM. In control samples, the inhibitor was added together with [^{3}H]carnitine, as described in the inhibitor stop procedure.[20] Using this methodology, the radioactivity diffusing through membranes in the presence of the inhibitor could be subtracted from that of the samples in which the inhibitor was added at the end of the transport reaction. For efflux experiments, after a first passage on Sephadex G-75, proteoliposomes were incubated with 0.05 mM [^{3}H]carnitine for 1 h at 25 °C to fill proteoliposomes with the radioactive substrate. The proteoliposomes were then passed on a second Sephadex G-75 column, and the efflux was started by adding buffer alone [10 mM Pipes (pH 7.0)] (uniport) or 2 mM unlabeled carnitine (antiport) to aliquots of 100 μL of proteoliposomes. In control samples, the inhibitor (1.5 mM NEM) was added at time zero. The decrease in radioactivity inside the proteoliposomes was measured at various times by stopping the transport with NEM. Finally, the [^{3}H]carnitine that remained in the external space was removed by gel filtration on Sephadex G-50, and the radioactivity in the internal space was detected. The transport activities were then measured. Details of the procedures are described in ref 19.

Effect of Fluorescein 5-Maleimide on CAC Mutants. Experiments that included labeling with fluorescein 5-maleimide (FM) were performed in the proteoliposomes in the absence or presence of external 2 mM carnitine. The mutant proteins were exposed to 10 μM FM for 5 s at 25 °C, and then the reaction was quenched by adding 10 mM DTE. After this treatment, the proteoliposomes were passed through Sephadex G-75 columns and ultracentrifuged; then, the pellets were separated via SDS−PAGE and analyzed by fluorescence detection (MultiImager, Bio-Rad) and Coomassie Blue staining.

Other Methods. Protein concentrations was estimated using on-gel densitometry as previously described.[21] The extent of incorporation of protein into liposomes was measured as previously described.[7] The homology model of the human CAC was built using the structure of the bovine ADP/ATP carrier[1]as a template by the computer application Swiss PDB Viewer.[22]

RESULTS

In a manner different from that of most mitochondrial carriers that behave as obligatory antiporters, the CAC catalyzes, besides antiport, also a uniport reaction with a slower rate[15,18] (see Figure 1). Under the conditions used in the experiments, the transport rate of the antiport, calculated from the time course, as the product of k, the first-order rate constant, and the transport at equilibrium, was 28.0 nmol mg^{-1} min^{-1} while the rate of the uniport was 9.3 nmol mg^{-1} min^{-1}, with a ratio of 3.0 between the two modes of transport. These conditions were chosen after comparison with the ratio between catalytic efficiencies (V_{max}/K_m) of antiport and uniport transports for the WT (3.1 ± 0.82). This parameter was evaluated as previously described[16] from experiments in which the transport rate was measured as a function of a variable (0.2−2 mM) carnitine concentration in the presence (antiport) or absence (uniport) of an intraliposomal substrate. A similar ratio (2.5) was calculated for the transport at equilibrium between the two modes (not shown, but see Figure 1a). We have analyzed the effect of mutations on the trans-stimulation by the counter-substrate (antiport) with respect to the WT. For a suitable comparison among the mutants, which exhibit very different specific activities,[16] transport is referred, as a percentage, to the maximal uniport of each protein [100% (Figure 1)]. According to this representation, the transport rates in the antiport and uniport modes of the WT were 22.0 and 7.3% min^{-1}, respectively, leading to the same ratio obtained between the specific activities (Table 1).

Table 1. Uptake Activities of Recombinant WT and Mutant CAC Proteins in the Antiport and Uniport Modes (see Experimental Procedures)[a]

protein	uniport (% min^{-1})	antiport (% min^{-1})	antiport/uniport ratio
WT	7.3 ± 0.40	22 ± 2.3[b]	3.0 ± 0.36
H29A	5.6 ± 1.5	21 ± 2.7[b]	3.8 ± 1.11
D32A	1.6 ± 0.25	2.3 ± 0.37	1.4 ± 0.32
K35A	1.4 ± 0.30	1.4 ± 0.45	1.0 ± 0.39
K35R	4.4 ± 0.84	16 ± 1.2[b]	3.6 ± 0.75
K97A	4.8 ± 0.30	7.2 ± 1.5	1.5 ± 0.33
G121A	5.8 ± 0.61	20 ± 1.5[b]	3.4 ± 0.45
E132A	1.5 ± 0.20	5.3 ± 0.19[b]	3.5 ± 0.49
K135A	5.5 ± 0.85	15 ± 0.30[b]	2.7 ± 0.43
R178A	2.2 ± 0.40	4.9 ± 1.1[c]	2.2 ± 0.64
D179A	nd[d]	nd[d]	nd[d]
D179E	5.4 ± 0.33	17 ± 1.4[b]	3.1 ± 0.32
E191A	1.7 ± 0.10	5.2 ± 0.12[b]	3.1 ± 0.19
K194A	2.5 ± 0.20	5.1 ± 1.6[c]	2.0 ± 0.66
D231A	1.4 ± 0.26	2.4 ± 1.3	1.7 ± 0.98
K234A	1.4 ± 0.25	5.8 ± 0.40[b]	4.1 ± 0.79
R275A	nd[d]	nd[d]	nd[d]
E288A	1.2 ± 0.056	2.9 ± 1.0[c]	2.4 ± 0.84

[a]Rates of uptake have been calculated as the product of k, the first-order rate constant, and the percent of radioactivity taken up at equilibrium derived from interpolation of the data depicted in Figures 1−3 and 5 in a first-order rate equation. The antiport/uniport rate ratios are reported. The data represent means ± SD of four independent experiments. [b]Statistical significance of antiport with respect to uniport of each protein estimated by a Student's t test ($p < 0.01$). [c]Statistical significance of antiport with respect to uniport of each protein estimated by a Student's t test ($p < 0.05$). [d]Not determined.

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The analyses were performed also by following [^{3}H]carnitine efflux in the presence (antiport) or absence (uniport) of external unlabeled carnitine. Efflux activities are expressed as the percentage of initial intraliposomal radioactivity for each mutant. Rates of efflux (% min^{-1}) were calculated as the product of k, the first-order rate constant, and the percent of effluxed radioactivity. An antiport/uniport ratio of 2.5 (Table 2) or transport at equilibrium of 1.9 (not shown but see Figure 1b) was calculated for WT. Charged residues belonging to the matrix and cytosolic networks and residues located in the middle of the cavity involved in substrate recognition have been mutated. In uptake experiments, the rates of uniport and antiport measured for most mutants are lower than those of the WT, according to the impairment in catalytic efficiency previously reported.[16] While the reactions are measured as efflux, the rates measured for mutants are somewhat comparable to those of the WT for methodological reasons, because uptake is measured with an external substrate concentration below saturation (0.1 mM) and lower than that used in efflux experiments [2 mM (see Experimental Procedures and ref 18)]. Results showing the effect of each mutation on time courses of [^{3}H]carnitine uptake and efflux in proteoliposomes are grouped in Figures 1−3. Couples of mutated residues undergoing ion pair interactions in the matrix gate (D32 and K135, K35 and D231, and E132 and K234) and in the cytosolic gate (K97 and E191, and K194 and E288) and the critical amino acids of the central binding center (R178 and H29)[13,16] that are conserved in the members of the CAC subfamily (Figure 4) are grouped in each panel of Figures 1−3. The additional substitution of G121 has been tested, G121 being homologous to G119 of CIC, which was previously found to be critical for the antiport function of that transporter.[23] Among the first couple of residues, the substitution of K135 did not cause appreciable variations in trans-stimulation by internal carnitine, compared to that of the WT (Figure 1a). Indeed, the antiport rate was significantly higher than the uniport one with a ratio of 2.7 (Table 1), similar to that of the WT. On the other hand, the substitution of residue D32 (Figure 1a) led to a decrease in the antiport rate with an antiport/uniport ratio of 1.4 (Table 1). When the transport was studied by efflux experiments, the results observed for both K135A and D32A mutants mostly reproduced those obtained in the uptake experiments (Figure 1b and Table 2). The substitution of residues of the second couple, K35 or D231, led to a loss or a reduction of coupling with a ratio between antiport and uniport of 1.0 or 1.7, respectively (Figure 1c and Table 1). It is noteworthy that when the ratio between catalytic efficiencies of antiport and uniport transport was measured for K35A, as was done for the WT (see above), a ratio of 1.2 ± 0.41 was found, which confirmed the critical role of this residue. When the efflux was being measured, similar behaviors were observed (Figure 1d). In the case of the third couple, E132 and K234, the antiport rates were always significantly higher than the uniport rates when they were measured both as uptake and as efflux with ratios comparable to or even higher than those of the WT (Figure 1e,f and Tables 1 and 2). With regard to the charge pairs of the cytosolic gate, K97A showed impaired antiport rates that were not significantly different from those of uniport in both uptake and efflux mode, while its charge pair E191A did not show any variation with respect to that of the WT (Figure 2a,b). Also, in the case of K97A, the ratio between catalytic efficiencies of antiport and uniport transport, measured as for the WT (see above), was evaluated (0.89 ± 0.31) and found to be in agreement with the data listed in Table 1. The K194A or E288A substitution resulted in antiport/uniport rate ratios similar to that of the WT when measured in uptake, while lower values were observed with the efflux procedure (Figure 2c,d). Mutants H29A and R178A showed behaviors similar to that of the WT (Figure 3a,b). The experiments could not be performed on the D179A and R275A mutants that were nearly inactive.[16] In addition, some conservative mutants were also tested such as K35R, D179E, R178K, and (not shown) R275K. In these mutants, the antiport/uniport ratio (Figure 5 and not shown) was similar to that of the WT. Also, mutant G121A exhibited behavior overlapping that of the WT (Figure 3).

Table 2. Efflux Activities of Recombinant WT and Mutant CAC Proteins in the Antiport and Uniport Modes (see Experimental Procedures)[a]

protein	uniport (% min^{-1})	antiport (% min^{-1})	antiport/uniport ratio
WT	1.5 ± 0.25	3.7 ± 0.43[b]	2.5 ± 0.50
H29A	1.29 ± 0.36	4.0 ± 0.70[b]	3.1 ± 1.02
D32A	1.0 ± 0.15	1.4 ± 0.45	1.4 ± 0.50
K35A	1.1 ± 0.20	1.1 ± 0.24	1.0 ± 0.28
K35R	1.6 ± 0.47	3.7 ± 0.48[b]	2.3 ± 0.14
K97A	1.1 ± 0.37	1.5 ± 0.42	1.4 ± 0.60
G121A	1.1 ± 0.15	4.7 ± 0.45[b]	4.3 ± 0.71
E132A	0.7 ± 0.10	2.9 ± 0.53[b]	4.1 ± 0.96
K135A	1.8 ± 0.47	3.9 ± 0.60[b]	2.2 ± 0.66
R178A	1.8 ± 0.42	3.9 ± 0.50[b]	2.2 ± 0.64
D179A	nd[c]	nd[c]	nd[c]
D179E	1.4 ± 0.10	2.2 ± 0.28[b]	1.6 ± 0.23
E191A	1.1 ± 0.20	3.1 ± 0.51[b]	2.8 ± 0.69
K194A	1.5 ± 0.20	2.1 ± 0.51	1.4 ± 0.39
D231A	1.6 ± 0.21	1.6 ± 0.24	1.0 ± 0.20
K234A	1.1 ± 0.25	5.1 ± 0.35[b]	4.6 ± 1.1
R275A	nd[c]	nd[c]	nd[c]
E288A	1.7 ± 0.40	1.9 ± 0.20	1.1 ± 0.29

[a]Rates of efflux have been calculated as the product of k, the first-order rate constant, and the percent of effluxed radioactivity derived from interpolation of the data depicted in Figures 1−3 and 5 in a single-exponential decay equation. The antiport/uniport rate ratios are reported. The data represent means ± SD of four independent experiments. [b]The statistical significance of antiport with respect to uniport of each protein estimated by a Student's t test ($p < 0.01$). [c]Not determined.

The influence of the most critical mutation, K35A, on the conformational states of the transporter was probed, using chemical reagents specific for SH groups of Cys residues for labeling both the inward (m-state) and outward (c-state) conformations of the carrier. Interaction of the transporter with NEM that binds specifically C136 only in the m-state[7,24] was tested on mutant K35A, in comparison to the WT (Figure 6). Binding was detected as inhibition of the transport in the absence or presence of substrate carnitine, as previously described.[7] NEM binding led to ∼50% inhibition of transport on the WT. The presence of carnitine during the incubation did not influence the transport function of the control and reduced the level of inhibition by NEM to ∼30% (Figure 6). These results correlated well with previously published data.[7] The treatment with NEM of the mutant K35A led to inhibition of ∼60%. However, in a manner different from that of the WT, the presence of substrate had nearly no influence on the extent of inhibition by NEM. To label the c-state of the CAC, a

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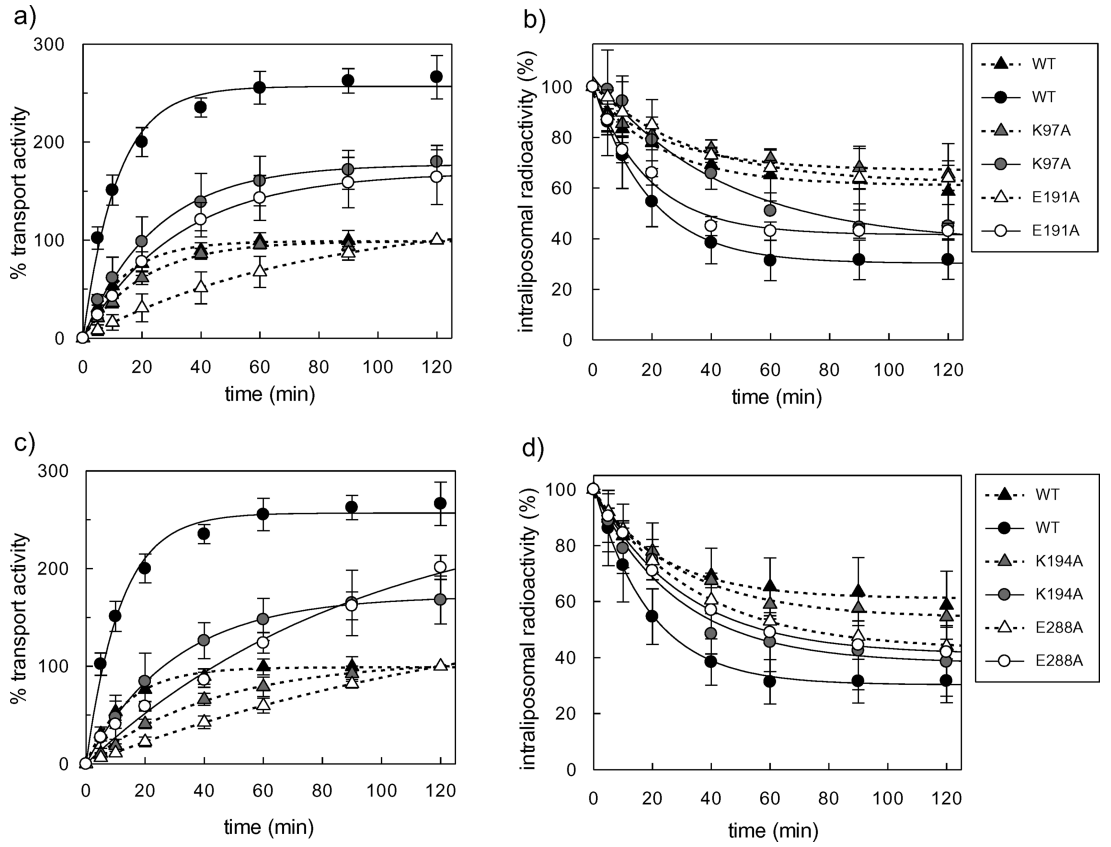

Figure 2. Antiport and uniport activity of WT and mutant CAC proteins of the cytosolic gate charged residues. (a and c) Uptake was started by the addition of 0.1 mM [^{3}H]carnitine to proteoliposomes with (antiport) or without (uniport, dotted lines) 5 mM carnitine. (b and d) Efflux of 5 mM [^{3}H]carnitine from prelabeled proteoliposomes measured as described in Experimental Procedures by adding buffer alone (uniport, dotted lines) or 2 mM unlabeled external carnitine (antiport). Data are reported as the percentage of the transport at equilibrium in the uniport mode of the WT. The data represent means ± SD of at least four independent experiments.

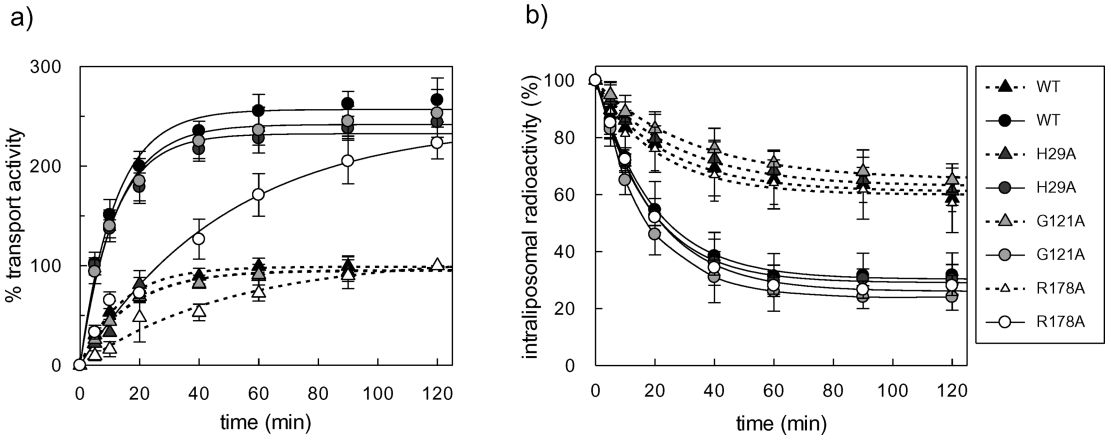

Figure 3. Antiport and uniport activity of WT CAC and selected mutants of core residues. (a) Uptake was started by the addition of 0.1 mM [^{3}H]carnitine to proteoliposomes with (antiport) or without (uniport, dotted lines) 5 mM carnitine. (b) Efflux of 5 mM [^{3}H]carnitine from prelabeled proteoliposomes measured as described in Experimental Procedures by adding buffer alone (uniport, dotted lines) or 2 mM unlabeled external carnitine (antiport). Data are reported as the percentage of the transport at equilibrium in the uniport mode of the WT. The data represent means ± SD of at least four independent experiments.

membrane impermeable fluorescent dye that targets Cys residues[25] has been used. For a specific labeling of the c-state, C89 and/or C283, located in the cytosolic cavity (Figure 7), had to be targeted. Because FM binds also to C136 and C155 (but not C58 or C23)[9] (unpublished results), a double-Cys mutant lacking C136 and C155 has been constructed to target specifically the c-state. The additional K35A mutation has been introduced into this double mutant, to test the effect of this residue on the conformation of the protein. The K35A/C136A/C155A mutant had very low but measurable transport activity (see below). Then, FM labeling was tested on the mutants C136A/C155A and K35A/C136A/C155A. A single batch of proteoliposomes containing internal carnitine was prepared and divided into two aliquots for assaying FM labeling

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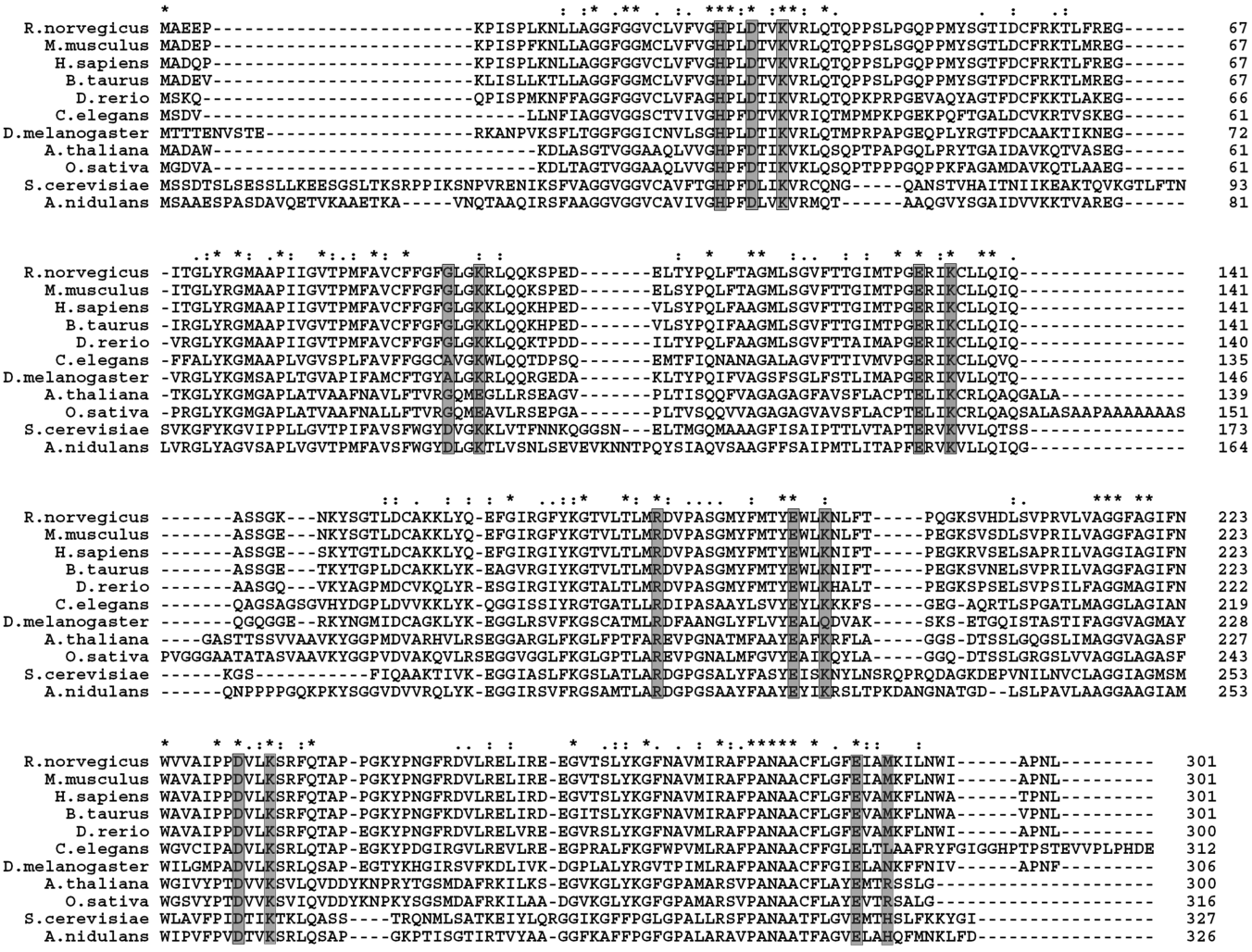

Figure 4. Alignment of proteins of the mitochondrial carnitine carrier subfamily. CAC proteins (NP446417 from *Rattus norvegicus*, NP000378 from *Homo sapiens*, NP568670 from *Arabidopsis thaliana*, NP014743 from *Saccharomyces cerevisiae*, AJ011563 from *Aspergillus nidulans*, NP065266 from *Mus musculus*, XP001253588 from *Bos taurus*, NP957153 from *Danio rerio*, NP501223 from *Caenorhabditis elegans*, NP477221 from *Drosophila melanogaster*, and NP001065471 from *Oryza sativa*) were aligned using ClustalW. Identities are indicated by asterisks and conservative or highly conservative substitutions indicated by periods or colons, respectively. Charged residues undergoing ion pair interactions and critical amino acids of the central binding center are highlighted in gray.

in the absence (uniport) or presence (antiport) of external carnitine. Both mutants were labeled by FM, demonstrating that FM targets C89 and/or C283, i.e., the c-state of the protein (see also Figures 7 and 9). A substrate-induced increase in the extent of labeling was observed in mutant C136A/C155A. On the other hand, no variation in labeling was observed, upon addition of an external substrate, in the case of K35A/C136A/C155A (Figure 8A–C). To exclude the possibility that substitution of K35 might result in the opposite orientation of the protein in the bilayer, the occurrence of complete inhibition by FM of K35A and K35A/C136A/C155A mutants in comparison with that of the WT was tested. Both K35A and K35A/C136A/C155A were virtually completely (100%) inhibited at 42 and 80 μM FM, respectively, like the WT, which was inhibited at 50 μM FM. Because FM is a membrane impermeant reagent, these data indicate a nearly 100% insertion right-side-out with respect to the native protein. The higher concentration of FM required for inhibition of the K35A/C136A/C155A mutant is related to the lack of C136 and C155, which contribute together with C89 and C283 to the binding and inhibition by FM.

The effect of external carnitine was investigated with respect to the transport function of mutant K35A/C136A/C155A in comparison to mutant C136A/C155A. Efflux of [^{3}H]carnitine from reconstituted proteoliposomes was measured in the absence or presence of an external substrate (Figure 8D). In the case of C136A/C155A, the addition of external carnitine stimulated the efflux rate like it did for the WT; almost no stimulation was observed with the K35A/C136A/C155A mutant, confirming that the antiport function was abolished by the mutation of K35 (Figure 8D).

DISCUSSION

Structural proof of the reaction mechanism of mitochondrial carriers is still not available. Several computational analyses have been performed[17,26,27] to gain information about the most plausible mechanisms, in some cases in combination with site-directed mutagenesis.[16,23,28−30] Some hypotheses about the possible conformational changes underlying the transport process have recently been made on the basis of the structures of the bovine and yeast ADP/ATP transporters.[2] On the basis of all the transport mechanisms proposed previously,

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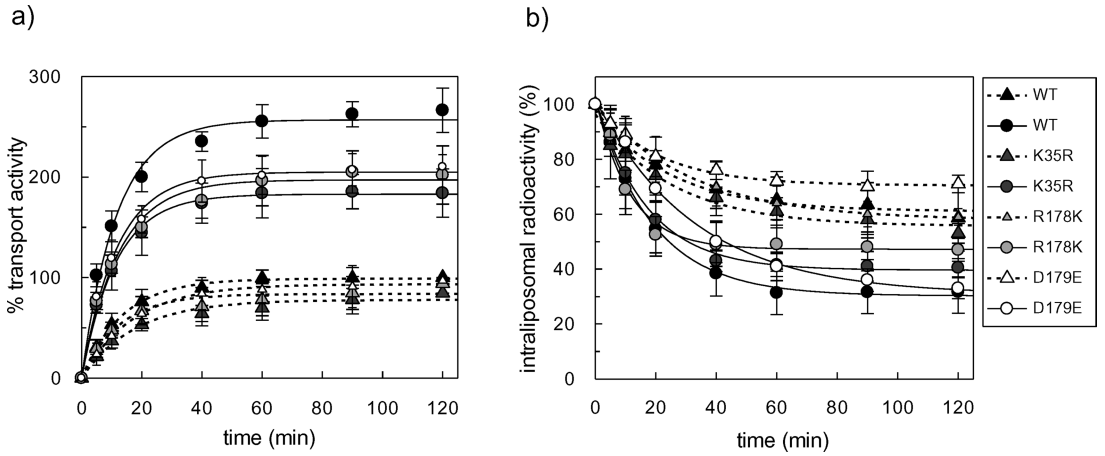

Figure 5. Antiport and uniport activity of WT CAC and selected conservative mutants. (a) Uptake was started by the addition of 0.1 mM [^{3}H]carnitine to proteoliposomes with (antiport) or without (uniport, dotted lines) 5 mM carnitine. (b) Efflux of 5 mM [^{3}H]carnitine from prelabeled proteoliposomes measured as described in Experimental Procedures by adding buffer alone (uniport, dotted lines) or 2 mM unlabeled external carnitine (antiport). Data are reported as the percentage with respect to WT uniport. The data represent means ± SD of at least four independent experiments.

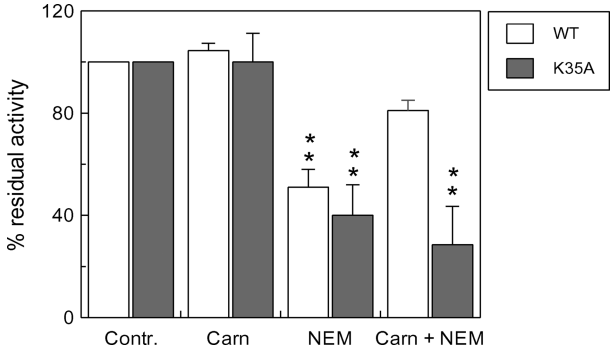

Figure 6. Effect of external substrate on the inhibition by NEM of WT CAC and its K35A mutant. Proteoliposomes containing 5 mM internal carnitine were incubated with 20 μM NEM for 2 min at 25 °C in the absence (uniport) or presence (antiport) of 5 mM external carnitine.

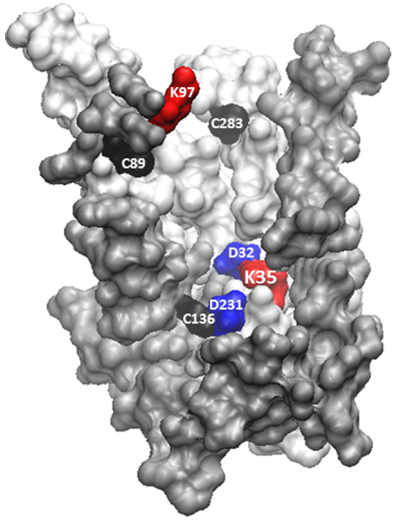

Figure 7. Side view of the central cavity in the CAC structural homology model. Cross section of the CAC homology model in the c-state[37] highlighting the central cavity. The structure is represented using the molecular visualization program VMD. α-Helices H2 (left) and H5 (right) in the foreground are colored gray. α-Helices H1 (bending from left to right) and H6 (middle) in the background are colored white. H3 and H4 have been omitted to show the cavity. Cys and critical Lys and Asp residues are indicated.

mitochondrial carriers can be considered single-binding center gated pores.[31] According to this model, binding of an external substrate to the transporter in the cytosolic conformation (c-carrier-substrate$_{ex}$) induces energy changes that allow the transition of the transporter to the opposite matrix conformation (m-carrier-substrate$_{ex}$) and release of the substrate on the matrix side, and vice versa (see Figure 9). The rate of transition of the unbound transporter (m-carrier) to the initial conformation (c-carrier) is negligible or slower than that of the substrate-loaded transporter; i.e., the transition is facilitated by the presence of the countersubstrate on the opposite side. Most mitochondrial carriers function by an antiport mechanism that is formally the result of a coupling of two uniport reactions in opposite directions.[32] The transport process involves the formation of an occluded state in which the substrate is hidden to both the cytosolic and the matrix sides, as previously hypothesized on the basis of data obtained with the ADP/ATP transporter[30] or on the basis of computational analysis (see Figure 9 and refs 27 and 33) and suggested for the yeast CIC on the basis of a transport assay and site-directed mutagenesis.[23] From a kinetic point of view, coupling of two uniport reactions in monomeric transporters[34−36] corresponds to a ping-pong mechanism. This kinetic mechanism has been well documented for CAC.[9] The transporter, which is different from most mitochondrial carriers, also catalyzes the uniport reaction in both directions, which has been found both in intact mitochondria[37] and in liposomes reconstituted with the purified or recombinant transporter from rat or human,[15,18] respectively. Therefore, the two half-reactions actually occur, and this finding correlates well with the hypothesis that mitochondrial transporters with lower energy barriers of the m- or c-gates may function as uniporters.[17] Indeed, the CAC has only four, instead of six, charged residues in the c-network.[16] In this work, the role of specific amino acid residues in CAC-mediated antiport has been investigated. The data described highlight the fact that four residues, of 14, including those of the matrix/cytosolic gates and substrate binding core, play a role in the molecular mechanism of antiport, i.e., the coupling

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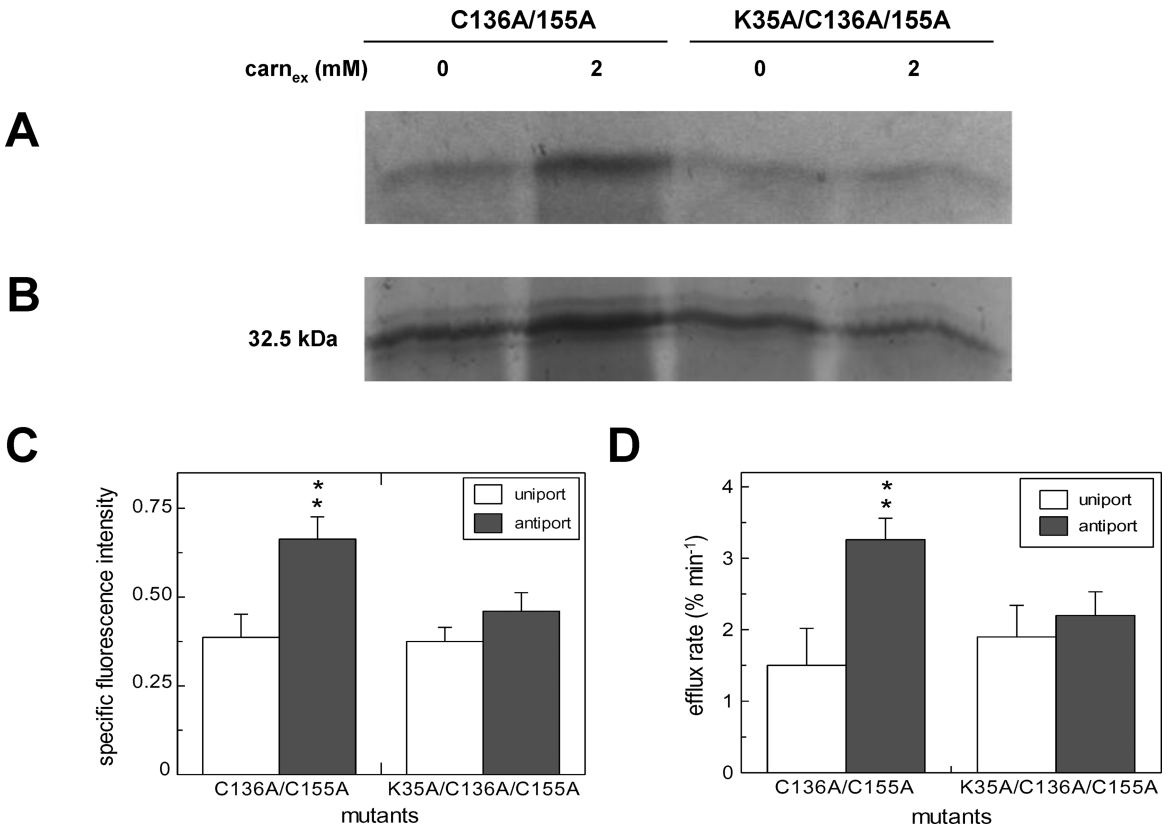

Figure 8. Effect of an external substrate on fluorescein 5-maleimide (FM) labeling of CAC mutants C136A/C155A and K35A/C136A/C155A. Proteoliposomes containing 5 mM internal carnitine were incubated with 10 μM FM for 5 s at 25 °C in the absence (uniport) or presence (antiport) of 2 mM external carnitine. The reaction was then quenched by adding 10 mM DTE. (A) Fluorescence of the UV-illuminated SDS−PAGE gel. (B) Coomassie Blue staining of the same gel. (C) Specific fluorescence, i.e., fluorescence/protein ratio measured by densitometric analysis of panels A and B, of the mutant proteins. Data are reported as a percentage and represent means ± SD of four independent experiments. (D) Efflux at 30 min of 5 mM [^{3}H]carnitine from proteoliposomes reconstituted with the indicated CAC mutants. Data are reported as a percentage with respect to the C136A/C155A uniport and represent means ± SD of four independent experiments. Significantly different from data for C136A/C155A uniport as estimated by the Student's t test (*p < 0.05; **p < 0.01).

of uniport reactions in opposite directions. K35 of the matrix network is the most critical. Its substitution with an uncharged residue led to a complete loss of antiport function (Figure 1 and Table 1). The charge of K35 is very important for its function as demonstrated by the fact that, as opposed to the K35A mutation, the conservative K35R mutation did not affect either the antiport or uniport function. D32 and D231 of the matrix network and K97 of the cytosolic network are also important for antiport. Other residues involved in transport function, whose substitution strongly impairs the catalytic efficiency of CAC, such as E132, K135, R178, K234, and E288 do not play any specific role in the antiport function. Mutants lacking more than one critical residue were inactive except for D32A/K35A and K35A/D231A. These mutants showed behavior that overlapped with that of K35A, i.e., loss of antiport function (not shown). The impairment of function was previously described for the CIC upon mutation of G119 and R181. Substitution of the homologous residues of CAC, i.e., G121 and R178, did not lead to any variation in either antiport or uniport function (see Figure 3). Moreover, the homologue of K35 in the CIC, i.e., K37, was not involved in the antiport function but only in substrate specificity.[23] On this basis, it might be hypothesized that residues that are critical for coupling are less conserved than those involved in substrate recognition.[2,16,23] According to previously published data,[9,18] a fundamental role in coupling the uniport counter-reactions is played by the countersubstrate. The relationship between the critical K35 residue and the countersubstrate was investigated by studying the consequences of the K35 mutation in experiments in which the m-state or the c-state has been labeled with NEM or FM, respectively (Figures 6 and 8). On one hand, our data indicate that the countersubstrate facilitates the release of the substrate coming from the opposite compartment (Figure 9A, sketches 1 and 4). This effect is probably due to stabilization of the cytosolic or matrix-open conformation (Figure 9A, sketches 2 and 5). In the absence of a countersubstrate, the overall process is impaired because the transition of the empty carrier, from 5 to 1 (Figure 9B), is slower. On the other hand, mutation of K35 abolished the capacity of the cytosolic (or matrix) countersubstrate to accelerate the transition of the transporter conformation from the m-state to the c-state or vice versa, in agreement with the transport experiments (Figure 1). In this respect, K35, together with the other three residues (D32, K97, and D231), may play a role in the formation of the occluded state and also in sensing the presence of the countersubstrate. A network of weak bonds can be hypothesized, which realizes a cross-talk among the different residues involved in the antiport mechanism. Indeed, in the absence of only one of the critical residues, coupling is strongly impaired or, in the most critical K35A mutant, completely lost. All the data concur with the model in which transport occurs via different states of the transporter[34] (Figure

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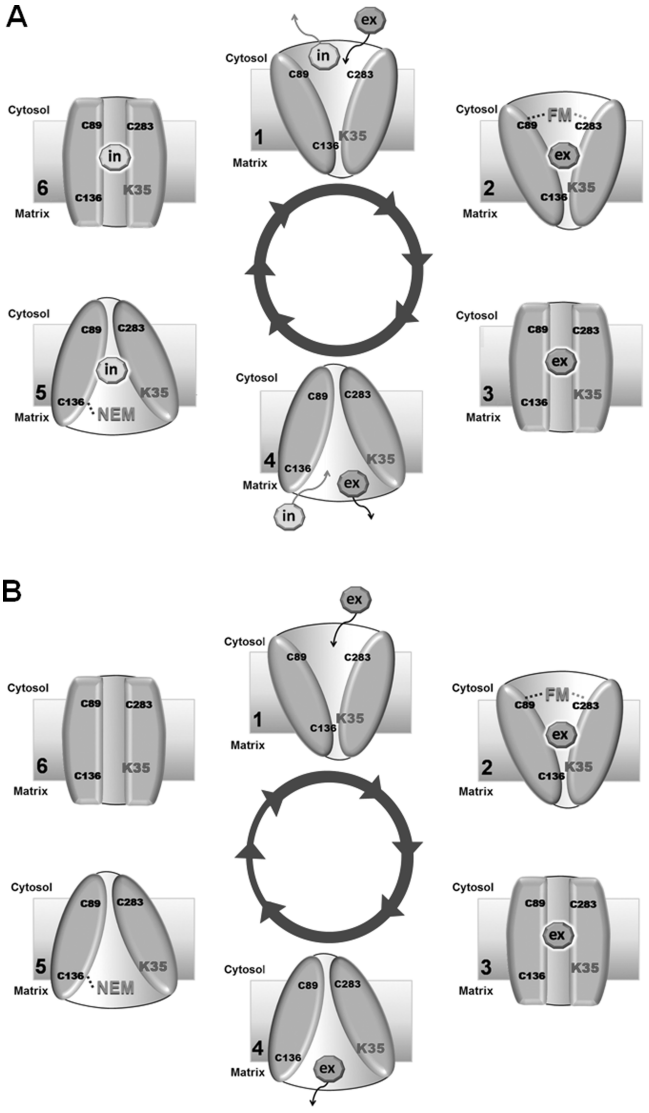

Figure 9. Sketch of the possible transport cycle of the CAC and role of the substrates. (A) The states of the transporter, during antiport reaction, are depicted: (1) c-state in which the internal substrate is released, (2) c-state with the external substrate bound [this conformation can be labeled by FM that can bind to C89 (gray dotted line) or C283 (blue dotted line)], (3) occluded state with an external substrate bound, (4) m-state in which the external substrate is released, (5) m-state with the internal substrate bound (this conformation can be labeled by NEM, which binds to C136), and (6) occluded state with an internal substrate bound. (B) The states of the transporter, during uniport reaction, are depicted: (1) c-state in which the external substrate is approaching, (2) c-state with the external substrate bound [this conformation can be labeled by FM that can bind to C89 or C283 (dotted lines)], (3) occluded state with an external substrate bound, (4) m-state in which the external substrate is released, (5) empty m-state that can be labeled by NEM, and (6) empty occluded state. Cys residues involved in labeling and the most critical K35 residue are highlighted.

9). This model correlates well with the ping-pong kinetic mechanism found for the CAC,[9,38] which predicts the formation of binary, not ternary, complexes of the carrier and substrate. The higher uniport rate that is found specifically in efflux mode with the K194A and E288A mutants can be explained by the additional reduction of the cytosolic network energy, as previously hypothesized for other transporters on the basis of computational data.[16]

In conclusion, on the basis of previously published results and the results presented here, it can be assessed that the antiport mode of transport, typical of mitochondrial carriers, results from coupling of uniport reactions in opposite directions mediated by specific amino acid residues. In the absence of the countersubstrate, the energy required to open the occluded state and then to release the substrate in the opposite compartment strongly increases. Coupling is generated by few selected residues, which seem not to be conserved in the transporters of the family. When the most critical K35 or one of the other critical residues is substituted, coupling of the two uniport reactions in opposite directions is lost or impaired. The possibility that other residues, which have not been analyzed in this work, may play some additional and/or minor roles in coupling cannot be excluded.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CAC, carnitine/acylcarnitine carrier; CIC, citrate carrier; DTE, 1,4-dithioerythritol; NEM, *N*-ethylmaleimide; FM, fluorescein 5-maleimide; Pipes, 1,4-piperazinediethanesulfonic acid; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WT, wild type.

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