Biochimica et Biophysica Acta 1817 (2012) 697–704

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Identification by site-directed mutagenesis of a hydrophobic binding site of the mitochondrial carnitine/acylcarnitine carrier involved in the interaction with acyl groups

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ARTICLE INFO

Article history:
Received 14 November 2011
Received in revised form 30 January 2012
Accepted 7 February 2012
Available online 15 February 2012

Keywords:
Carnitine
Acylcarnitine
Mitochondria
Liposome
Mutagenesis
Transport

ABSTRACT

The role of hydrophobic residues of the mitochondrial carnitine/acylcarnitine carrier (CAC) in the inhibition by acylcarnitines has been investigated by site-directed mutagenesis. According to the homology model of CAC in cytosolic opened conformation (c-state), L14, G17, G21, V25, P78, V82, M85, C89, F93, A276, A279, C283, F287 are located in the 1st (H1), 2nd (H2) and 6th (H6) transmembrane α-helices and exposed in the central cavity, forming a hydrophobic half shell. These residues have been substituted with A (or G) and in some cases with M. Mutants have been assayed for transport activity measured as [^{3}H]carnitine/carnitine antiport in proteoliposomes. With the exception of G17A and G21M, mutants exhibited activity from 20% to 100% of WT. Among the active mutants only G21A, V25M, P78A and P78M showed Vmax lower than half and/or Km more than two fold respect to WT. Acylcarnitines competitively inhibited carnitine antiport. The extent of inhibition of the mutants by acylcarnitines with acyl chain length of 2, 4, 8, 12, 14 and 16 has been compared with the WT. V25A, P78A, P78M and A279G showed reduced extent of inhibition by all the acylcarnitines; V25M showed reduced inhibition by shorter acylcarnitines; V82A, V82M, M85A, C89A and A276G showed reduced inhibition by longer acylcarnitines, respect to WT. C283A showed increased extent of inhibition by acylcarnitines. Variations of Ki of mutants for acylcarnitines reflected variations of the inhibition profiles. The data demonstrated that V25, P78, V82, M85 and C89 are involved in the acyl chain binding to the CAC in c-state.

1. Introduction

The mitochondrial carnitine/acylcarnitine carrier (CAC) plays an essential role in the ß-oxidation of fatty acids [1]. After the initial studies in intact mitochondria, the function of the CAC has been extensively investigated by reconstituting the purified protein in liposomes [2–4]. In proteoliposomes the CAC catalyses efficient carnitine/carnitine homologous and carnitine/acylcarnitine heterologous antiports and, in the absence of counter-substrate, a 10 times slower carnitine uniport. The transporter is functionally asymmetrical and is inserted in the proteoliposomal membrane in a right-side out orientation as compared to mitochondria. The functional asymmetry of the transporter correlates well with its physiological role. Indeed, the CAC shows, on the external cytosolic side, higher affinity towards acylcarnitines than for free carnitine; furthermore the affinity for acylcarnitines is higher respect to the matrix side. These kinetic properties cause preference for transport of acylcarnitines, respect to carnitine, into the mitochondrial matrix, where the acyl groups are transferred to CoA and subjected to β-oxidation. The free carnitine released in the matrix is translocated by the antiport reaction towards the cytosol [1,4]. The amino acid sequence of the CAC possesses all the features of the mitochondrial carrier protein family [5], i.e., three repeated segments of about 100 amino acids, each containing the signature motif PX[DE]XX[RK]. As in the case of the other members of the family, in the CAC sequence six hydrophobic transmembrane segments (H1–H6) can be identified by hydropathy analysis. These

Abbreviations: DTE, dithioerythritol; NEM, *N*-ethylmaleimide; Pipes, 1,4-piperazinediethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WT, wild-type; CAC, carnitine/acylcarnitine carrier; ANC, adenine nucleotide carrier; PIC, phosphate carrier; CIC, citrate carrier

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doi:10.1016/j.bbabio.2012.02.007

segments are connected by five hydrophilic loops, two of which are exposed towards the cytosolic side (h_{23}, h_{45}) and three towards the matrix side (h_{12}, h_{34}, h_{56}) of the protein; the three matrix loops are much larger than the cytosolic ones. The CAC is present in several species. Among the various unicellular or multicellular organisms in which it has been identified by biochemical or genomic approaches the CAC is encoded in *Homo sapiens* by the gene SLC25A20 [6] that maps to chromosome 3p21.31 [7], in *Saccharomyces cerevisiae* by the gene CRC [8] and in *Aspergillus nidulans* by the gene acuH [9]. The rat and the human CAC have been over-expressed in *Escherichia-coli* and the recombinant proteins have been reconstituted in liposomes. The reconstituted proteins show functional and kinetic properties very similar to those of the native rat transporter [10,11]. Combining site-directed mutagenesis, chemical targeting and bioinformatics, the CAC has been characterized from the structural point of view. The homology structural model of the CAC was built using the ANC structure as template [12]. This model has been validated by experimental evidence demonstrating that C136 is located in the water-filled cavity. Furthermore, it was found that C136 and C155, which are distantly located in the primary structure, can become closer during some steps of the transport cycle demonstrating the occurrence of conformational changes of the protein induced by the interaction of the substrate with the transporter and involving both hydrophilic and hydrophobic residues [12,13]. In analogy with the ANC, in the structural model of the CAC, six conserved charged residues of the three conserved motifs constitute a salt bridge network, closing the central cavity of the transporter towards the matrix side. This structural feature has been confirmed by site-directed mutagenesis studies, which, moreover, demonstrated that K35 and E132, which are involved in the salt-bridge network, play a major role in opening and closing the matrix gate. The amino acid residues R275, D179 and R178 of CAC have been found to be involved in the binding of the zwitterionic substrate carnitine [14], while the residue H29 plays a role in positioning of the substrate during the translocation [15]. However, the physiological substrates that enter the mitochondrial matrix are acylcarnitines [1,16]. These substrates have large hydrophobic moieties, which should bind hydrophobic amino acid residues. Using site-directed mutagenesis together with molecular modeling and docking, the residues that are implied in the binding of acyl moieties of acylcarnitines have been identified.

2. Experimental procedures

2.1. Materials

Sephadexes G-50, G-75 and G-200 were purchased from Pharmacia, L-[methyl-^{3}H]carnitine from Amersham, egg-yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), Pipes, Triton X-100, cardiolipin, L-carnitine, acylcarnitines (acetyl-DL-carnitine, butyryl-L-carnitine, octanoyl-DL-carnitine, lauroyl-DL-carnitine, myristoyl-DL-carnitine, palmitoyl-L-carnitine and *N*-dodecanoylsarcosine (sarkosyl) from Sigma-Aldrich. All other reagents were of analytical grade.

2.2. Site-directed mutagenesis, overexpression and isolation of the CAC proteins

The previously constructed pMW7-WTratCAC recombinant plasmid was used to introduce the mutations in the CAC protein [17]. The amino acid replacements were performed with complementary mutagenic primers using the overlap extension method [18] and the High-Fidelity PCR System (Roche). The PCR products were purified by the QIAEX II Gel Extraction Kit (QIAGEN), digested with NdeI and HindIII (restriction sites added at the 5' end of forward and reverse primers, respectively) and ligated into the pMW7 expression vector. All mutations were verified by DNA sequencing, and except for the desired base changes, all the sequences were identical to that of rat CAC cDNA. The resulting plasmids were transformed into *E. coli* C0214. Bacterial overexpression, isolation of the inclusion body fraction, solubilization and purification of the WT CAC and mutant CAC proteins were performed as described previously [10,17].

2.3. Reconstitution of WT and mutant CAC proteins in liposomes

The recombinant proteins were reconstituted into liposomes as described previously [10,17]. The concentration of intraliposomal carnitine was 15 mM. The external substrate was removed from proteoliposomes on Sephadex G-75 columns.

2.4. Transport measurements

Transport at 25 °C was started by adding 0.1 mM [^{3}H]carnitine to proteoliposomes and terminated by the addition of 1.5 mM NEM. In controls, the inhibitor was added together with the labeled substrate, according to the inhibitor stop method [19]. This strategy allows to subtract from the experimental samples the aliquot of radiolabeled carnitine diffusing through the liposomal membrane. Finally, the external substrate was removed by chromatography on Sephadex G-50 columns, and the radioactivity in the liposomes was measured [19]. The experimental values were corrected by subtracting control values. All of the transport activities were determined by taking into account the efficiency of reconstitution (i.e., the share of successfully incorporated protein).

2.5. Other methods

SDS-PAGE was performed according to Laemmli [20] as previously described [10]. The amount of recombinant protein was estimated on Coomassie blue-stained SDS-PAGE gels by the Bio-Rad GS-700 Imaging Densitometer equipped with the software Bio-Rad Multi-Analyst, using bovine serum albumin as standard. The extent of incorporation of the recombinant protein in liposomes was determined as described in Phelps et al. [21], with the modifications reported in ref. [17]. The homology model of the human CAC was built based upon the structure of the bovine ADP/ATP carrier [22] by using the computer application Swiss PDB Viewer [23]. The hydrophobicity of α-helices has been calculated as GRAVY (grand average of hydropathicity) [24] using the bioinformatic tool ProParamTool (http://www.expasy.ch/tools/protparam.html). Prediction of the position of the substrate palmitoylcarnitine in the protein binding site has been performed using the software ArgusLab [25].

3. Results

3.1. Bioinformatic analysis

To predict which of the hydrophobic amino acid residues of CAC could be involved in the interaction with the acyl moieties of acylcarnitines, a bioinformatic analysis has been performed. The grand average of hydropathicity (GRAVY) [24] has been calculated for each α-helix of the CAC. α-Helices H1 (GRAVY, 0.80), H2 (0.84), H5 (0.84) and H6 (0.91) are more hydrophobic than H3 (0.40) and H4 (−0.16). Accordingly, the top view of the protein showing the residues protruding in the central cavity, highlights that most of the hydrophobic residues are grouped in an half shell, constituted by H1, H2 and H6 (Fig. 1). The hydrophobic half shell might contain the binding sites for acyl groups of acylcarnitines. This hypothesis has been evaluated using site-directed mutagenesis. Differently from CAC, hydrophobic residues protruding towards the cavity in transporters for charged substrates, such as the PIC, CIC and ANT are uniformly distributed among the six α-helices (not shown).

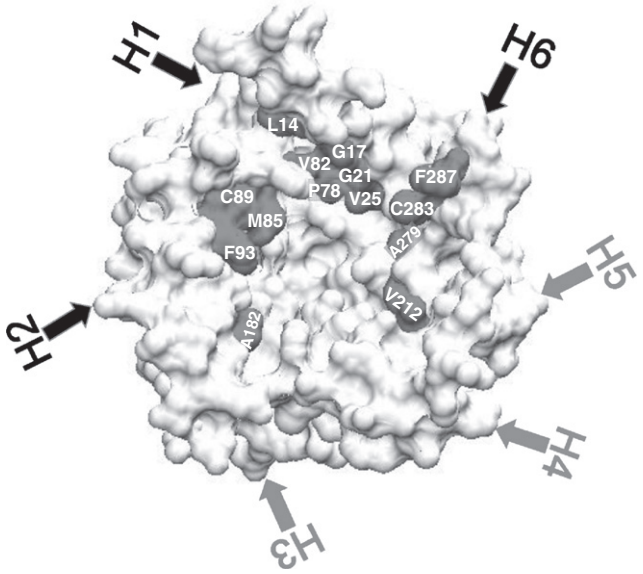

Fig. 1. Top view of CAC structural model. Surface representation in which the superficial residues protruding towards the central cavity are visible. The arrows, pointing to the cytosolic end of each transmembrane segment, indicate the orientation of α-helices H1–H6. The visible hydrophobic residues are highlighted in dark gray and labeled. The homology structural model has been represented using the molecular visualization program VMD.

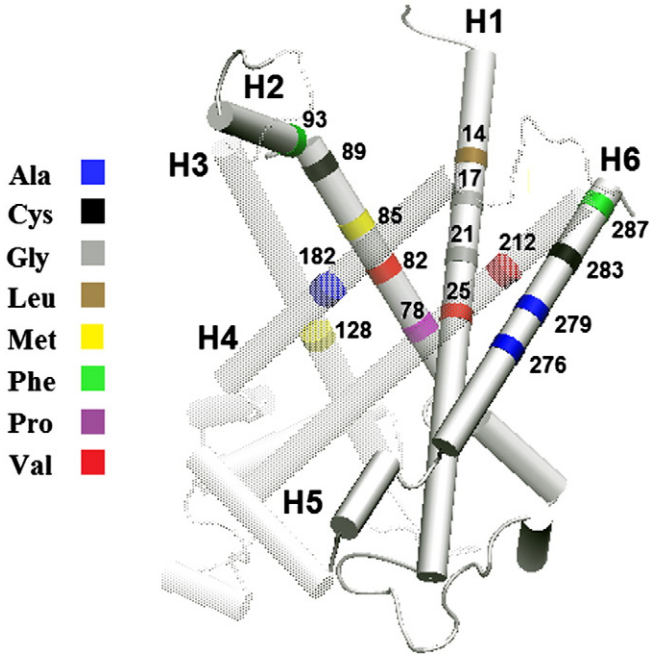

Fig. 2. Diagram of the CAC structure depicting the location of the mutated residues facing the central cavity. The transmembrane segments (H1–H6) of the CAC are represented by cylinders; segments H3, H4 and H5 are transparent. The residues are highlighted by colors as indicated.

3.2. Functional characterization of mutant CAC

On the basis of the bioinformatic predictions three sets of residues have been substituted: (1) hydrophobic residues of H1, H2 and H6 protruding in the central cavity, which may be hypothetically involved in acylcarnitine binding (Fig. 2); (2) hydrophobic residues of H3, H4 and H5 facing the cavity, which are not expected to be critical for acylcarnitine binding (Fig. 2); (3) some hydrophobic residues of H1, H2 and H6 that do not protrude towards the cavity that have been mutated as negative controls. Residues have been mutated with A or G and in some cases with M. The effect of the mutations of each transmembrane segment, in terms of variations of the GRAVY index, is reported in supplementary Table S1. In all the cases variations below 15% of the index were observed, except in the case of the most hydrophilic H4. The overall hydrophobicity of the entire protein was not significantly influenced (less than 7%) by each of the mutations (not shown). The mutant proteins, overexpressed in *E. coli*, have been reconstituted in liposomes for functional characterization. The efficiency of incorporation of the protein into the liposomal membrane has been tested as previously described [14]. No significant differences of incorporation were observed for the various mutants (not shown) as previously found for several other mutants of CAC [14,15,17]. The transport activity of each mutant has been tested in proteoliposomes as time course of [^{3}H]carnitine/carnitine antiport, in comparison with the WT (Fig. 3). Most of the mutants of hydrophobic residues protruding towards the central cavity (Fig. 3A–D, white symbols), exhibited transport activities comparable to or above 70% of that of WT, such as L14A, V25A and V25M (H1; Fig. 2 A), V82A, V82M, M85A and F93A (H2; Fig. 3 B), A276G, C283A and F287A (H6; Fig. 3 C), A182G (H4; Fig. 2 D), V212A (H5; Fig. 3 D). Some mutants showed lower activities, with transport at 30 min ranging from 20% to 50% of the WT activity, such as G21A (H1; Fig. 3 A), P78A, P78M and C89A (H2; Fig. 3 B), A279G (H6; Fig. 3 C); only G17A and G21M (H1; Fig. 3 A), M128A (H3; Fig. 3 D) were inactive (transport activity lower than 5% of the WT). The mutants of residues that do not protrude towards the cavity (Fig. 3, black symbols), i.e., V22A and C23A of H1 (Fig. 2 A), M75A and F86A of H2 (Fig. 3 B), A281G of H6 (Fig. 3 C), showed transport function similar or above 70% of that of WT, with the exception of L24A (H1), which exhibited transport activity at 30 min, of 40% respect to the WT. Thus, with few exceptions, the mutations performed did not cause strong impairment of transport function. On the contrary, substitutions of the residues with polar ones led, in general, to strong impairment of function (not shown).

3.3. Profile of inhibition of CAC mutants by acylcarnitines

Acylcarnitines behave as competitive inhibitors towards carnitine since they bind to the same site of the transporter (refs. [10,16] and see Fig. 5 below). Thus, the effect of the mutations on the interaction of CAC with acylcarnitines of various lengths can be tested by studying their inhibition profile. For this purpose the residual activity, i.e., the residual [^{3}H]carnitine/carnitine antiport in the presence of acylcarnitines of various lengths respect to the control (in the absence of inhibitor) has been measured. The residual activity of the mutants has been compared with the residual activity of the WT, in the presence of each acylcarnitine (Fig. 4). For a reliable estimation of the variations, the concentration of each inhibitor has been chosen (see legend of Fig. 4) to obtain, in the WT, residual activities of roughly 50%. Due to methodological reasons, consistent fluctuations (S.D. values) affected the experimental data in these experiments. Thus, a statistical analysis was performed to evaluate significant differences respect to the WT. Fig. 4A shows the effect of the substitutions with A (and M in the case of V25) of residues of H1, which face into the central cavity. While for the mutants L14A and G21A no significant variations were found, in the case of V25A a significant increase of residual activity, i.e., decrease of inhibition was observed with all acylcarnitines with very little difference from C2 to C14. In the mutant V25M, the decrease of inhibition became more evident with acylcarnitines from C4 to C8 and disappeared with longer carbon chains.

Fig. 4B and C shows the effect of the substitutions with A (and/or Met) of residues of H2 that face into the central cavity. The substitution of P78 with A caused extensive decrease of inhibition, respect to the WT, with all the acylcarnitines from 48% for C2 to more than

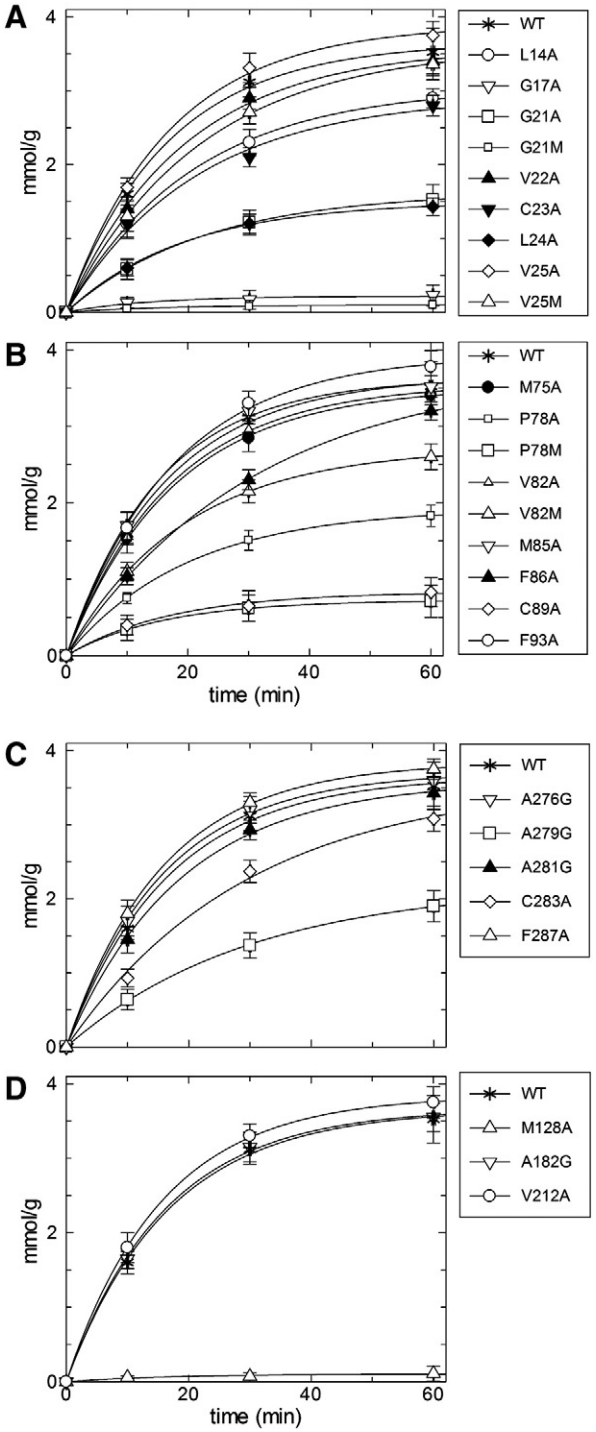

Fig. 3. Time course of [^{3}H]carnitine uptake in liposomes reconstituted with recombinant CAC proteins. Transport was started by the addition of 0.1 mM [^{3}H]carnitine to proteoliposomes containing 15 mM carnitine and stopped at the indicated times: (A) mutants of H1, (B) mutants of H2, (C) mutants of H6, (D) mutants of other helices. In each panel the data relative to the WT CAC are shown with those relative to the mutants, as indicated in the legends. White symbols or black symbols refer to mutants of residues, which protrude or do not protrude, respectively, towards the central cavity of CAC structural model. The data represent means ± SD of at least three independent experiments.

90% for C8, being higher than 60% for all the other acylcarnitines (Fig. 4 B). The substitution of P78 with Met, instead of A, caused similar but less remarkable variations especially in the case of C16. The mutation V82A caused significant increase of residual activity, i.e., decrease of inhibition above 13%, only in the case of acylcarnitines from C8 to C16. The substitution of V82 with M instead of A caused a more evident decrease of inhibition with the same acylcarnitines. In the case of M85A (Fig. 4 C), similar to the mutants of V82, no effect was observed with C2 and C4, but strong effects (more than 50% decrease inhibition) with acylcarnitines with longer chains. Again similar variations, even though less pronounced, were observed for the C89A mutant. The F93A mutant did not show significant effects. Fig. 4D shows the effect of the substitutions with A (or G in the cases in which A was the native residue) of residues of H6, which face towards the central cavity. The substitution A276G caused significant decrease of inhibition only with acylcarnitines from C12 to C16 ranging from 13% to 45%. The mutant A279G showed decrease of inhibition by all acylcarnitines. Surprisingly, the mutation C283A caused decrease of residual activity, i.e., an increase of inhibition, respect to the WT, with acylcarnitines from C8 to C16. A smaller and less significant increase of inhibition was observed with shorter (C2 and C4) acylcarnitines. Whereas the mutant F287A did not show significant variations.

The mutants of residues of H1, H2 and H6 which do not face the central cavity, V22A, C23A, L24A, M75A, F86A and A281G (Fig. 4E and F) as well as the mutants of the hydrophobic residues, facing the central cavity, located on H4 (A182G) and H5 (V212A) (Fig. 4 F) did not exhibit significant variation of inhibition by acylcarnitines. The only exception was observed for the effect of C2 on A182G: in this case a significant decrease of residual activity, i.e., increase of inhibition was observed. The charged residues that were previously found to interact with free carnitine were also tested for variations of inhibition by acylcarnitines. With the exception of H29 [15], no significant variations were found in all the other cases (not shown). It has to be stressed, however, that for the residues D179, R275, K35 and E132 only conservative mutants could be analyzed since other mutants were inactive [14].

3.4. Kinetics of the CAC mutants

The Vmax and Km for carnitine have been measured for the mutants of residues that face the central cavity (Table 1). Vmax could not be determined for G17A and G21M and was strongly impaired in G21A, V25M and P78M, which showed values lower than half respect to the WT. Concerning the Km values, only G21A, P78A and P78M showed Kms more than two fold that of the WT. Thus, the strongly impaired mutants resulted to be G21A, V25M and P78M with catalytic efficiency (Vmax/Km) lower than 20% of WT. All the other mutants showed smaller variations respect to the WT, indicating that most of mutations did not influence significantly the binding and translocation of free carnitine.

The effect of the substitutions on the kinetic parameters of the CAC for acylcarnitines of C2, C8 and C16 was evaluated for some of the mutants, which showed significant variations of the inhibition profile (see Fig. 4). To obtain half saturation constants for acylcarnitines, Ki values were derived from experiments in which the effect of acylcarnitines was evaluated at various [^{3}H]carnitine concentrations. In Fig. 5 the patterns of inhibition of the WT (Fig. 5 A) and the mutant M85A (Fig. 5 B), as an example, by acylcarnitines (C2, C8 and C16) are reported. The acylcarnitines clearly behaved as competitive inhibitors in agreement with previous data [10,16]; the same inhibition pattern was observed for all the other mutants (not shown). The Ki values of the WT and mutants derived from the inhibition kinetic experiments are reported in Table 2. The lowest Ki value of the recombinant WT CAC was found for C16. It increased about three times for C8, and the Ki for C2 was more than one order of magnitude higher than that for C16. These data indicated that the transporter exhibits higher affinity for longer acylcarnitines in agreement with previous data obtained with the native CAC purified from rat liver mitochondria [16]. The mutant V25A showed higher Ki for acylcarnitines respect to the WT; the strongest

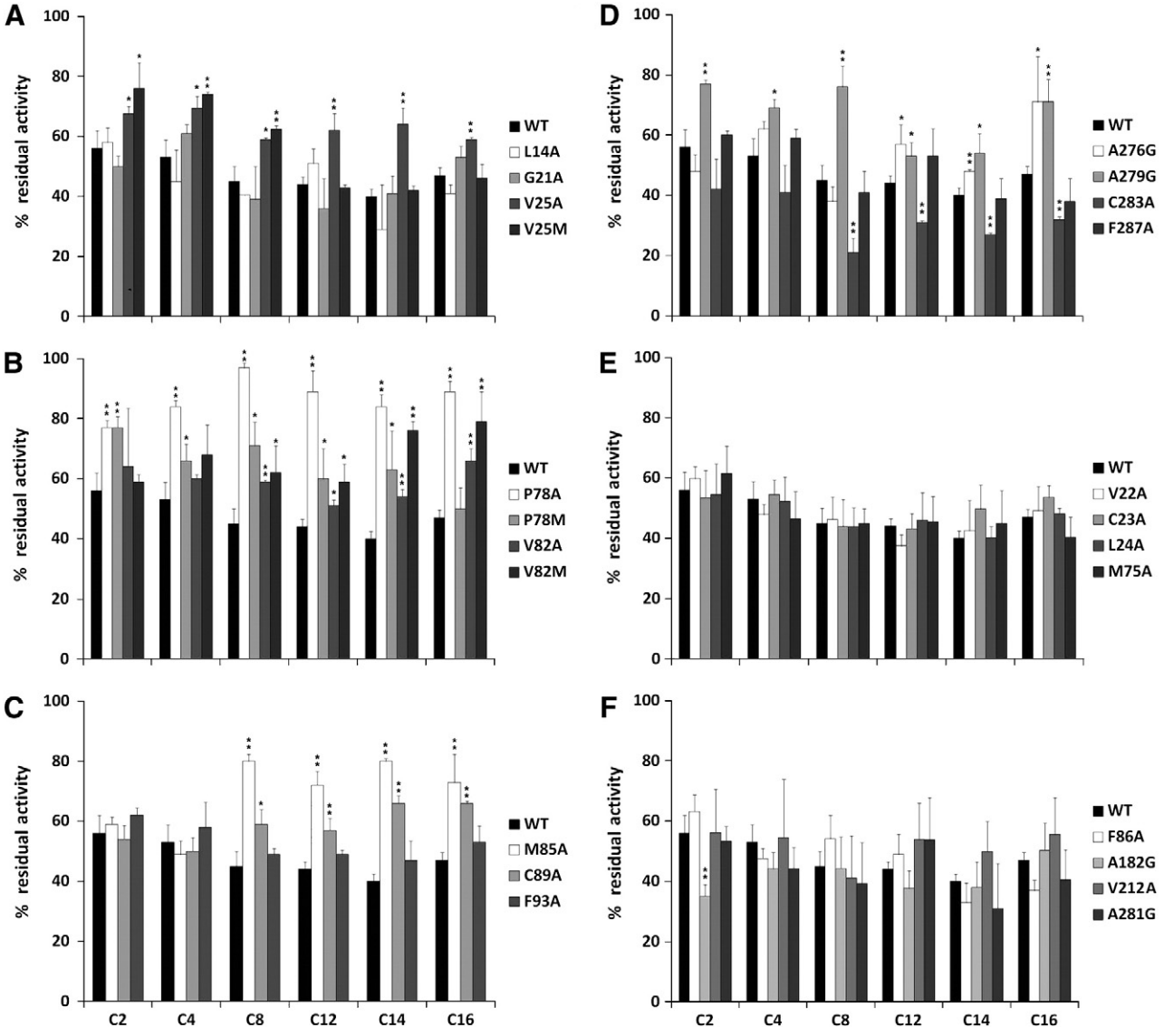

Fig. 4. Inhibition profile of CAC mutants by acylcarnitines. The carnitine acyl derivatives with the indicated (*n*) carbon chain length were added to the reconstituted proteoliposomes together with 0.1 mM [^{3}H]carnitine. The concentrations used for acylcarnitines were 0.2 mM acetylcarnitine (C2) and butyrylcarnitine (C4); 0.025 mM octanoylcarnitine (C8); 0.01 mM lauroylcarnitine (C12), myristoylcarnitine (C14) and palmitoylcarnitine (C16). After 15 min the transport was stopped as indicated in Materials and Methods. Percent age of residual activity are reported in comparison with the WT. In each panel (A–F) the colors related to the different mutants are indicated. The data represent means ± S.D. of three independent experiments. Significantly different from the WT as estimated by the Student's *t* test ($^*p<0.05$, $^{**}p<0.01$).

increment was observed in the case of C2. Among the mutants of H2, P78A showed the largest increment of Ki for C2 and C8 respect to the WT, whereas the Ki could not be measured for C16, in agreement with the almost complete loss of inhibition (Fig. 3 B). V82A and C89A showed strong Ki increase for C16 and smaller increases for C2 and C8; M85A showed strong increase of Ki for C8 and C16, in respect to the WT. In the case of H6 strong Ki increments for the three acylcarnitines were observed in the mutant A279G, whereas, in the mutant A276G only the Ki for C16 increased; C283A showed reductions of Ki for C8 and C16, in respect to the WT. The variations of Ki values mostly correlated with the variations of inhibition respect to the WT, shown in Fig. 4. To predict the structural model of the interactions among the critical residues and palmitoylcarnitine, a docking analysis was performed using ArgusLab [25]. The model obtained is shown in Fig. 6. The hydrophobic interactions among methylene groups of the acyl chain and residues V25, P78, V82, M85, C89 of H1 and H2 are depicted.

4. Discussion

The structure of the CAC in the cytosolic state (c-state) was previously constructed [11] by homology modeling using as template the structure of the ANC in the c-state obtained by X-ray crystallography [22]. In the c-state conformation, the protein is constituted by a bundle of six transmembrane α-helices, which delimit a central cavity closed towards the matrix by a network of three salt bridges. The relationships between the transporter and the substrate carnitine have been recently defined by site-directed mutagenesis coupled with bioinformatics. It has been found that R275, D179, K35, E132 and H29 are critical for the binding and translocation of free carnitine [14,15]. Physiologically CAC catalyses the transport of acylcarnitines with acyl groups of various lengths in exchange with carnitine. The transporter exhibits higher affinity (lower Ki) for longer acylcarnitines [10,16] (see also Table 2). On the basis of kinetic studies, it was demonstrated that the binding site for these molecules overlaps

Table 1
Kinetic constants of reconstituted CAC mutants in comparison with the WT. The Km and Vmax values were calculated from experiments in which the rates of carnitine/carnitine antiport were measured at different external substrate concentrations. Catalytic efficiencies are also reported (right column) as ratios Vmax/Km. Transport was started by the addition of 0.12–2.0 mM [^{3}H]carnitine to proteoliposomes reconstituted with the WT CAC or one of the indicated mutants and containing 15 mM carnitine. The transport time was 4 min. The data represent the means ± S.D. of four different experiments; nd, not detectable.

Protein	Km (mM)	Vmax (nmol/min/mg)	Vmax/Km (ml/min/mg)
WT	0.48 ± 0.091	1.8 ± 0.54	3.8
L14A	0.60 ± 0.12	1.0 ± 0.31	1.7
G17A	nd	nd	nd
G21A	0.96 ± 0.17	0.45 ± 0.080	0.47
G21M	nd	nd	nd
V25A	0.87 ± 0.077	1.7 ± 0.15	1.9
V25M	0.90 ± 0.059	0.62 ± 0.17	0.69
P78A	1.1 ± 0.10	1.6 ± 0.55	1.5
P78M	1.2 ± 0.15	0.72 ± 0.26	0.60
V82A	0.60 ± 0.15	1.5 ± 0.81	2.5
V82M	0.38 ± 0.083	2.0 ± 0.80	5.3
M85A	0.57 ± 0.15	2.1 ± 0.090	3.7
C89A	0.88 ± 0.11	1.0 ± 0.092	1.1
F93A	0.56 ± 0.082	1.1 ± 0.31	2.0
A276G	0.80 ± 0.10	1.7 ± 0.73	2.1
A279G	0.80 ± 0.22	0.93 ± 0.42	1.2
C283A	0.49 ± 0.070	1.4 ± 0.18	2.8
F287A	0.43 ± 0.11	1.2 ± 0.75	2.8

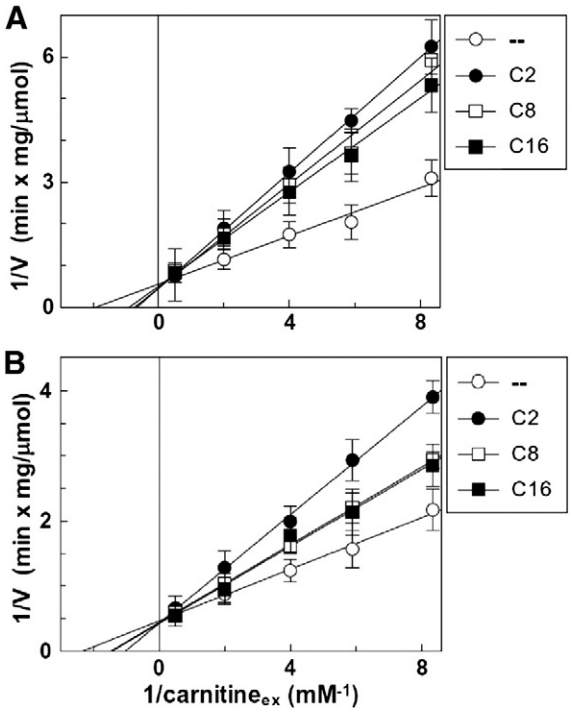

Fig. 5. Kinetic analysis of the inhibition of the recombinant transporters by acylcarnitines.The carnitine/carnitine antiport rate was measured, as described in Materials and Methods, adding [^{3}H]carnitine at different concentrations to proteoliposomes containing 15 mM carnitine, in the absence or in the presence of acylcarnitines. Experimental data for WT (A) and M85A (B) plotted according to Lineweaver–Burk as reciprocal transport rate vs. reciprocal carnitine concentrations in the absence (○) or in the presence of 250 µM acetylcarnitine (C2, ●), 25 µM octanoylcarnitine (C8, □) or 10 µM palmitoylcarnitine (C16, ■). The data represent means ± SD from at least three independent experiments.

that of carnitine [10,16] (see also Fig. 5). However, the acyl group binding site also contains hydrophobic amino acid side chains. To identify these residues, we have studied the alterations of the inhibition profile of acylcarnitines of various lengths caused by mutations of hydrophobic residues protruding into the central cavity of the transporter. The residues have been substituted with smaller hydrophobic residues: A or G, if the WT amino acid was A. These substitutions cause mainly changes in the volume of the residues, without significantly affecting the overall hydrophobicity of each α-helix (Table S1). Consequently, each of the substitutions would have the desired effect to abolish or reduce interactions among the amino acid side chains and the hydrophobic moiety of the acylcarnitines, causing variations of the inhibition. Eight of the mutated hydrophobic residues protruding into the cavity on the half shell made by H1, H2 and H6, influence the profile of sensitivity of the transporter towards acylcarnitines. Four of these residues are located on H2 indicating that it is the most critical transmembrane α-helix for acyl group recognition. A similar important role of the second transmembrane segment in the binding of acetylcarnitine was hypothesized on computational basis for the yeast mitochondrial carnitine transporter. In that case the residues predicted to be critical for acetyl group binding were I89 and F90 [26], which are homologues of M85 and F86 of the rat CAC. More recently, the importance of the second transmembrane segment had also been predicted for the mitochondrial CIC [27], which, however, recognizes anionic substrates. The mutations of critical residues in most cases had no or slight effect on the transport activity and Km for free carnitine, whereas affected the half saturation constants (Ki) for specific acylcarnitines (Table 2), indicating that the variations are mainly due to effect of mutations on the binding of the acyl chains to the transporter. Both variations of profile of inhibition and of Ki indicated that substitution of V25 and P78, which are located deeply in the central cavity (bottom residues), affected the interaction of the protein with all the acylcarnitines, i.e., acylcarnitines from C2 to C16 interact with the bottom residues. Whereas, substitution of V82, M85 and C89, which are located at about the midpoint of the membrane (middle residues), affected the interaction of longer acylcarnitines, i.e., only acylcarnitines from C8 to C16 interact with the middle residues. Some of the critical residues have also been substituted with Met, which is larger and, differently from A, increases the sterical hindrance. The effects observed in these mutants confirmed the conclusions drawn from the A mutants. Indeed the effect of mutation was similar respect to the A mutants. Only in the case of the bottom residues (P78M and V25M) longer acyl chains were less affected by the mutations. This last observation may be explained by the capacity of longer chains to assume different stable conformations, which adapt better than short chains to the increased sterical hindrance in the deeper part of the binding pocket. However, the effect observed in the P mutants, which is more evident than those of other residues could also be explained by a generalized alteration of structure and mobility of the protein. Indeed, it is well known that P residues affect the structure of α-helices.

Table 2
Ki values for acylcarnitines of CAC mutants. The Ki values were calculated from double-reciprocal plots obtained from rates of carnitine/carnitine antiport under variation of the external substrate concentration in the absence or presence of 250 µM acetylcarnitine (C2), 25 µM octanoylcarnitine (C8) or 10 µM palmitoylcarnitine (C16). Transport was started by the addition of 0.12–2.0 mM [^{3}H]carnitine to proteoliposomes reconstituted with the WT CAC or one of the indicated mutants and containing 15 mM carnitine. The transport time was 4 min. The data represent the means ± S.D. from three different experiments; n.d., not detectable.

Protein	Ki C2 (µM)	Ki C8 (µM)	Ki C16 (µM)
WT	120 ± 13	15 ± 1.9	5.4 ± 0.53
V25A	430 ± 34	43 ± 5.2	8.1 ± 0.90
P78A	640 ± 32	60 ± 3.2	nd
V82A	155 ± 9.6	26 ± 1.8	20 ± 2.8
M85A	190 ± 19	44 ± 1.9	26 ± 3.8
C89A	180 ± 47	23 ± 1.8	20 ± 1.0
A276G	124 ± 21	20 ± 4.1	28 ± 2.0
A279G	520 ± 94	35 ± 2.2	nd
C283A	110 ± 12	8.0 ± 1.2	3.0 ± 0.28

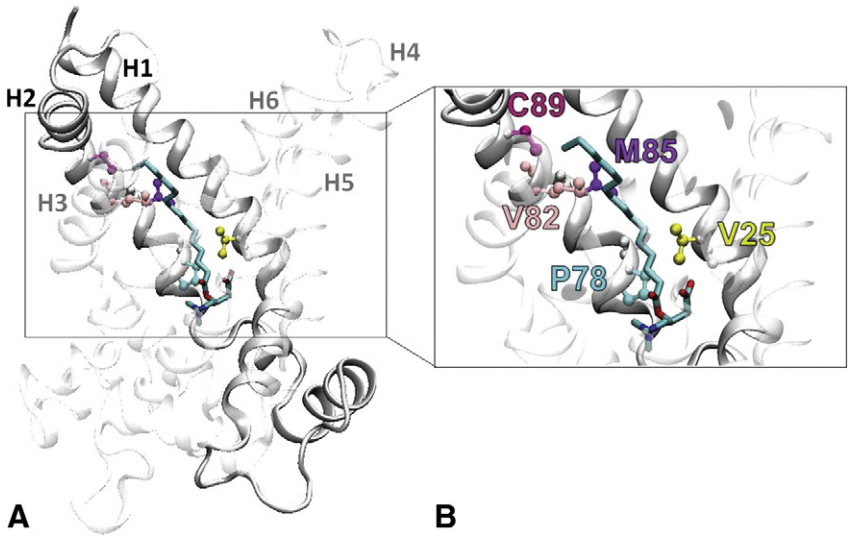

Fig. 6. Interaction of the CAC with palmitoylcarnitine. (A) Lateral view of the CAC structural model with bound palmitoylcarnitine. The transmembrane α-helices H1 (on the right) and H2 (on the left) are opaque whereas H3, H4, H5 and H6 are transparent. CAC residues involved in the interaction and the palmitoylcarnitine molecule are depicted in a ball and stick or tube representation, respectively. (B) Enlarged view of the residues interacting with palmitoylcarnitine.

The molecular model, which better fulfills the overall experimental results on H1 and H2 (Fig. 6), depicts palmitoylcarnitine as an example of acylcarnitines, oriented from the bottom to the edge of the cavity. This correlates well with the previous data describing the interaction of the carnitine moiety with residues K35, E132 and H29, which are located just below the hydrophobic bottom residues [14,15] and with the finding that substitution of residues that are located at a more superficial position (L14 and F93) did not affect the inhibition, i.e., the binding of acylcarnitines (see Fig. 4). It should be further considered that the central cavity of the transporter contains water molecules. Thus, also the more hydrophobic substrates such as longer acylcarnitines (C12–C16) would interact with some water molecules. Since acylcarnitines have been used at concentrations lower than their CMC [28], the molecules are in a monomer state in water. The solubility in this condition should be facilitated by formation of convoluted acyl chain structures that minimize the contact surface with water before the acyl chain binds to the hydrophobic binding site. The binding of the hydrophobic moiety does not constitute per-se the basis for substrate selectivity, which, indeed, is determined by the overall interactions, including binding of polar groups of carnitine to charged residues, as previously described [14]. The hydrophobic interaction protein-acyl group provides the molecular basis for increased affinity respect to free carnitine. Indeed, the number of interactions of amino acid residues with acyl chains increases with the chain length, explaining the higher affinity of CAC (lower Ki) for longer acylcarnitines [16] (see Table 2). G21 and G17 are not critical for the interaction with acyl groups. However, the lack of activity of the mutants G17A and G21M indicates that the two residues may play a role in the conformational changes involved in the translocation process, as previously predicted for the mitochondrial carrier family [29]. Apparently, the experimental data on H6 did not fit with those on H1 and H2. In particular, the substitution of C283 led to increase, not decrease, of inhibition by longer acylcarnitines. A plausible explanation for these findings may be that H6 interacts with the acylcarnitines when the carrier is in a conformation different from the c-state. Even though there are no experimental evidences yet, the existence of alternative conformations is in line with previous findings indicating that the binding of free carnitine to the CAC induces changes of conformation of the protein [13,14] and with the prediction that mitochondrial carriers undergo from c-state to m-state opened towards the matrix through a transition state [26,29–31]. The residues of H6 may have a role in this transition state. On the basis of the experimental data (Fig. 4 and Table 2), C283, getting closer to H2, may favor the displacement of the acyl moiety of acylcarnitines from H2 and release in the matrix. The anomalous increase of inhibition (binding) by longer acylcarnitines caused by mutation of C283 (Fig. 4 and Table 2) is thus explained by a decreased efficiency in displacing the acyl group from H2. The results of mutations of A276 and A279 with G may also be explained by a more generalized effect on the α-helix structure. This hypothesis correlates well with the previous finding that residues of H6 are involved in inter-helical interaction [11] and with the prediction of a cytosolic network of six residues, which are distant in the c-state and get close to each other when the protein is in the m-state [14,26]. Two of these six residues G94 and M291 are located on the H2 and H6, just above C89 and C283, respectively. Thus also C89 and C283 could get closer in the m-state. Interestingly, all the residues involved in the interaction with the acyl moiety are fully conserved only in the mammalian and zebra fish CAC proteins (not shown). This finding correlates well with the role of CAC in these organisms to provide mitochondria with medium-long acylcarnitines for β-oxidation. To our knowledge, this work represents the first description at molecular level of interaction between hydrophobic molecules and a member of the mitochondrial carrier family.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbabio.2012.02.007.

Acknowledgements

This work was supported by grants from the Ministero dell'Università e della Ricerca (MIUR), the Center of Excellence in Genomics (CEGBA), Apulia Region, the University of Bari, the Italian Human ProteomeNet No, RBRN07BMCT_009. The PON research project 01_00937 (MIUR).

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