Site-directed mutagenesis of charged amino acids of the human mitochondrial carnitine/acylcarnitine carrier: Insight into the molecular mechanism of transport

Nicola Giangregorio [a,b,1], Annamaria Tonazzi [a,b,1], Lara Console [a], Cesare Indiveri [c,*], Ferdinando Palmieri [a,b,*]

[a] Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, 70125 Bari, Italy
[b] CNR Institute of Biomembranes and Bioenergetics, via Orabona 4, 70125 Bari, Italy
[c] Department of Cellular Biology, University of Calabria, 87036 Arcavacata di Rende, Italy

ARTICLE INFO

ABSTRACT

The structure/function relationships of charged residues of the human mitochondrial carnitine/acylcarnitine carrier, which are conserved in the carnitine/acylcarnitine carrier subfamily and exposed to the water-filled cavity of carnitine/acylcarnitine carrier in the c-state, have been investigated by site-directed mutagenesis. The mutants were expressed in *Escherichia coli*, purified and reconstituted in liposomes, and their transport activity was measured as ^{3}H-carnitine/carnitine antiport. The mutants K35A, E132A, D179A and R275A were nearly inactive with transport activities between 5 and 10% of the wild-type carnitine/acylcarnitine carrier. R178A, K234A and D231A showed transport function of about 15% of the wild-type carnitine/acylcarnitine carrier. The substitutions of the other residues with alanine had little or no effect on the carnitine/acylcarnitine carrier activity. Marked changes in the kinetic parameters with three-fold higher Km and lower Vmax values with respect to the wild-type carnitine/acylcarnitine carrier were found when replacing Lys-35, Glu-132, Asp-179 and Arg-275 with alanine. Double mutants exhibited transport activities and kinetic parameters reflecting those of the single mutants; however, lack of D179A activity was partially rescued by the additional mutation R178A. The results provide evidence that Arg-275, Asp-179 and Arg-178, which protrude into the carrier's internal cavity at about the midpoint of the membrane, are the critical binding sites for carnitine. Furthermore, Lys-35 and Glu-132, which are very probably involved in the salt-bridge network located at the bottom of the cavity, play a major role in opening and closing the matrix gate.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The mitochondrial carnitine/acylcarnitine carrier (CAC) belongs to a large family of transport proteins called the mitochondrial carrier family [1, and refs. 2 and 3 for reviews]. Members of this family have common structural features; they are made up of three tandemly repeated homologous domains about 100 amino acids in length, and each repeat contains two hydrophobic segments (spanning the membrane as α-helices) and a characteristic sequence motif PX[D/E]XX[K/R]. CACs of several species have been identified by transport assays and/or by their high similarity to orthologs in other organisms [4–9]. Multiple sequence alignment of the CAC proteins revealed that they constitute a subfamily characterized by the RXXPANAAXF motif [9] and by specific triplets of amino acids [3,10]. This subfamily of mitochondrial carriers catalyzes carnitine/acylcarnitine and carnitine/carnitine antiport efficiently and, in the absence of counter-substrate, a slower carnitine uniport [11,12]. Both the homologous carnitine/carnitine exchange and the heterologous carnitine/acylcarnitine exchange occur via a ping-pong transport mechanism, implying the existence of two opposite conformations in which the substrate-binding site is alternately exposed towards the cytosol (c-state) or towards the matrix (m-state) [13 and refs. therein]. Physiologically, CAC is important in mammals as it allows the net import of fatty acyl units into mitochondria by catalyzing an exchange between extramitochondrial acylcarnitine and internal free carnitine. This transport reaction is essential for fatty acid β-oxidation, as confirmed by the identification of a disease known as carnitine carrier deficiency caused by mutations in the CAC gene [5 and 14 for a review]. Another member of the mitochondrial carrier family (accession no. NP001034444) was reported to accept long chain acylcarnitines [15]. However, this protein was later identified as an ornithine transporter [16].

The significant sequence conservation in the mitochondrial carrier family suggests that the main structural fold is similar for all carriers [17]. Until now, the 3D structure of only one member of the
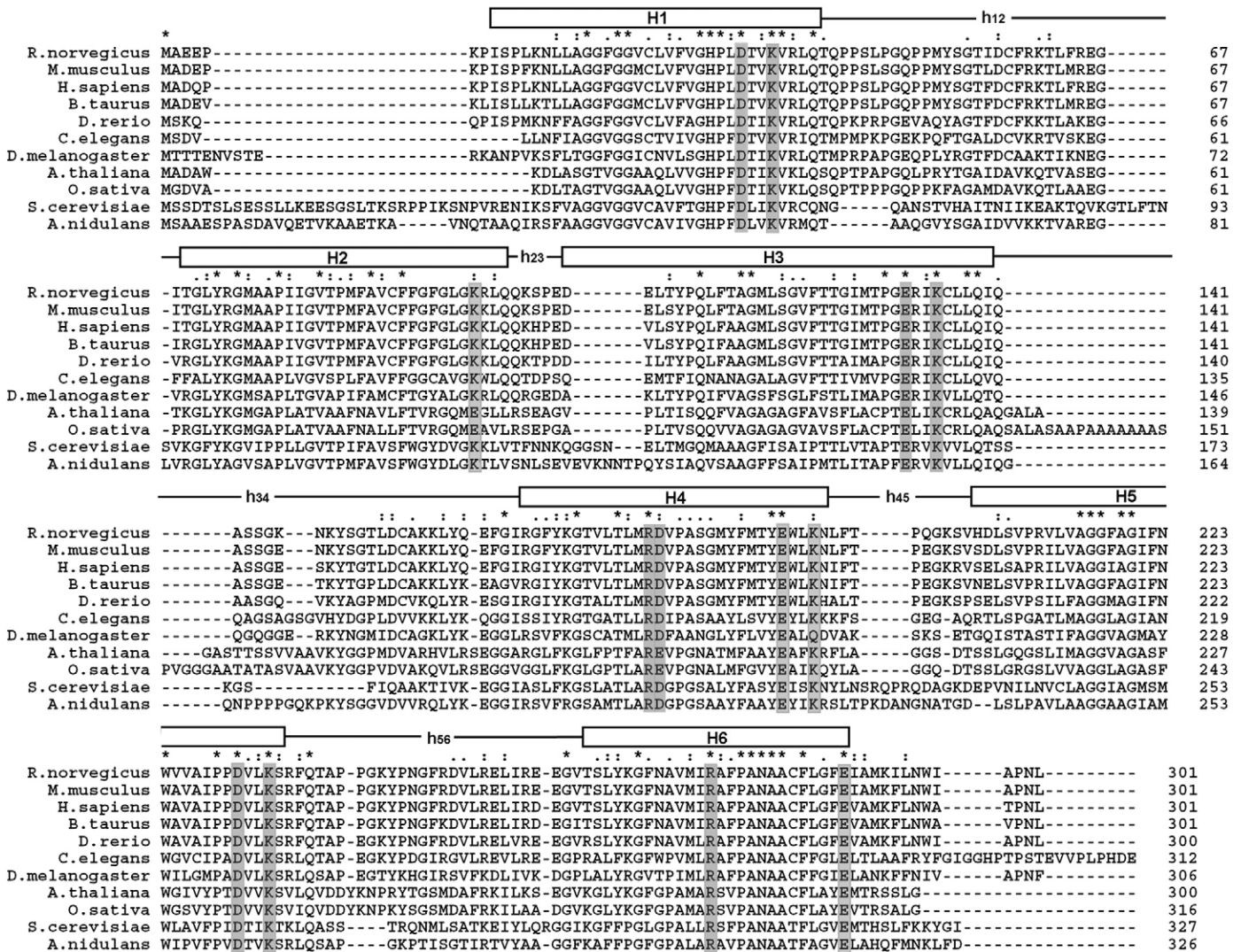
mitochondrial carrier family, the ADP/ATP carrier in complex with its powerful inhibitor carboxyatractyloside, has been determined [18]. This structure consists of six transmembrane α-helices (H1–H6) and three hydrophilic short α-helices (h₁₂, h₃₄ and h₅₆) parallel to the membrane plane. The H1–H6 bundle delimits a funnel-shaped cavity opened towards the cytosol and closed on the matrix side by a salt-bridge network (matrix gate) formed by the charged residues of the signature motifs. The crystal structure of the ADP/ATP carrier, which roughly corresponds to the c-state of mitochondrial carriers, has stimulated further research in the field of CAC. Indeed, the studies on CAC structure-function relationships already undertaken [19,20] have continued by combining homology modeling with site-directed mutagenesis and specific chemical labeling. It was found that Cys-136, CAC's major target of SH-blocking reagents, protrudes into the internal carrier cavity, and that the loop between H3 and H4 containing Cys-136 and Cys-155 undergoes conformational changes during the catalytic transport cycle [21,22]. Furthermore, it was found that His-29, which is conserved in all the members of the CAC subfamily and protrudes into the carrier cavity just above the matrix gate, participates in the substrate translocation mechanism [23]. Evidence was provided

that His-29 interacts via an H-bond with the alcoholic β-OH of carnitine or with the esterified β-O— of acylcarnitine, presumably playing a role in positioning the substrate prior to the opening of the matrix gate. In the present paper, the structure/function relationships of the Lys, Arg, Asp and Glu residues, which are exposed to the central cavity and conserved in the CAC subfamily, have been investigated. The experimental data shed light on the molecular mechanism of substrate transport and, in particular, on the binding of carnitine to CAC and opening/closing of the matrix gate.

2. Materials and methods

2.1. Materials

Sephadexes G-50, G-75 and G-200 were purchased from Pharmacia, l-[methyl-³H]carnitine from Amersham, egg-yolk phospholipids (l-α-phosphatidylcholine from fresh turkey egg yolk), Pipes, Triton X-100, cardiolipin, l-carnitine and N-dodecanoylsarcosine (sarkosyl) from Sigma. All other reagents were of analytical grade.

```
                                          H1                                 h12
              *              : :* .* .** . :. ***:* :**: *.                          . .:
R.norvegicus  MAEEP------------------------KPISPLKNLLAGGFGGVCLVFVGHPLDTVKVRLQTQPPSLPGQPPMYSGTIDCFRKTLFREG------  67
M.musculus    MADEP------------------------KPISPFKNLLAGGFGGMCLVFVGHPLDTVKVRLQTQPPSLSGQPPMYSGTLDCFRKTLMREG------  67
H.sapiens     MADQP------------------------KPISPLKNLLAGGFGGVCLVFVGHPLDTVKVRLQTQPPSLPGQPPMYSGTFDCFRKTLFREG------  67
B.taurus      MADEV------------------------KLISLLKTLLAGGFGGMCLVFVGHPLDTVKVRLQTQPPSLPGQPPMYSGTFDCFRKTLMREG------  67
D.rerio       MSKQ-------------------------QPISPMKNFFAGGFGGVCLVFAGHPLDTIKVRLQTQPKRPGEVAQYAGTFDCFKKTLAKEG------  66
C.elegans     MSDV-------------------------LLNFIAGGVGGSCTIVFFGHPFDTVKVRIQTMPMMPKPGEKPQFTGALDCVKRTVSKEG------  61
D.melanogaster MTTTENVSTE------------------RKANPVKSFLTGGFGGICNVLSGHPLDTIKVRLQTMPRPAPGEQPLYRGTFDCAAKTIKNEG------  72
A.thaliana    MADAW------------------------KDLASGTVGGAAQLVVGHPFDTIKVKLQSQPTPAPGQLPRYTGAIDAVKQTVASEG------  61
O.sativa      MGDVA------------------------KDLTAGTVGGAAQLVVGHPFDTIKVKLQSQPTPPPGQPPKFAGAMDAVKQTLAAEG------  61
S.cerevisiae  MSSDTSLSESSLLKEESGSLTKSRPPIKSNPVRENIKSFVAGGVGGVCAVFTGHPFDLIKVRCQNG-----QANSTVHAITNIIKEAKTQVKGTLFTN  93
A.nidulans    MSAAESPASDAVQETVKAAETKA-----VNQTAAQIRSFAAGGVGGVCAVIVGHPFDLVKVRMQT------AAQGVYSGAIDVVKKTVAREG------  81

               H2               h23            H3
              .:* * *: .*: *:.: *: *    : :        : *    ** :.. : : * * :* ** .
R.norvegicus  -ITGLYRGMAAPIIGVTPMFAVCFFGFGLGKRLQQKSPED-------ELTYPQLFTAGMLSGVFTTGIMTPGERIKCLLQIQ---------------  141
M.musculus    -ITGLYRGMAAPIIGVTPMFAVCFFGFGLGKKLQQKSPED-------ELSYPQLFTAGMLSGVFTTGIMTPGERIKCLLQIQ---------------  141
H.sapiens     -ITGLYRGMAAPIIGVTPMFAVCFFGFGLGKKLQQKHPED-------VLSYPQLFAAGMLSGVFTTGIMTPGERIKCLLQIQ---------------  141
B.taurus      -IRGLYRGMAAPIVGVTPMFAVCFFGFGLGKKLQQKHPED-------VLSYPQIFAAGMLSGVFTTGIMTPGERIKCLLQIQ---------------  141
D.rerio       -VRGLYKGMAAPIVGVTPMFAVCFFGFGLGKKLQQKTPDD-------ILTYPQLFAAGMLSGVFTTAIMAPGERIKCLLQIQ---------------  140
C.elegans     -FFALYKGMAAPLVGVSPLFAVFFGGCAVGKWLQQTDPSQ-------EMTFIQNANAGALAGVFTTIVMVPGERIKCLLQVQ---------------  135
D.melanogaster -VRGLYKGMSAPLTGVAPIFAMCFTGYALGKRLQQRGEDA-------KLTYPQIFVAGSFSGLFSTLIMAPGERIKVLLQTQ---------------  146
A.thaliana    -TKGLYKGMGAPLATVAAFNAVLFTVRGQMEGLLRSEAGV-------AGVPTELIKCRLQAQGALA---------------  139
O.sativa      -PRGLYKGMGAPLATVAAFNALLFTVRGQMEAVLRSEPGA-------PLTVSQQVVAGAGAGVAVSFLACPTELIKCRLQAQSSALASAAPAAAAAAAS  151
S.cerevisiae  SVKGFYKGVIPPLLGVTPIFAVSFWGYDVGKKLVTFNNKQGGSN---ELTMGQMAAAGFISAIPTTLVTAPTERVKVVLQTSS--------------  173
A.nidulans    LVRGLYAGVSAPLVGVTPMFAVSFWGYDLGKTLVSNLSEVEVKNNTPQYSIAQVSAAGFFSAIPMTLITAPFERVKVLLQIQG--------------  164

               h34                        H4               h45         H5
              :: . : : * .:* *:*: ..: : **  :       : ***. **
R.norvegicus  -------ASSGK---NKYSGTLDCAKKLYQ-EFGIRGFYKGTVLTLMRDVPASGMYFMTYEWLKNLFT-----PQGKSVHDLSVPRVLVAGGFAGIFN  223
M.musculus    -------ASSGE---NKYSGTLDCAKKLYQ-EFGIRGFYKGTVLTLMRDVPASGMYFMTYEWLKNLFT-----PEGKSVSDLSVPRILVAGGFAGIFN  223
H.sapiens     -------ASSGE---SKYTGTLDCAKKLYQ-EFGIRGIYKGTVLTLMRDVPASGMYFMTYEWLKNIFT-----PEGKRVSELSAPRILVAGGIAGIFN  223
B.taurus      -------ASSGE---TKYTGPLDCAKKLYK-EAGVRGIYKGTVLTLMRDVPASGMYFMTYEWLKNIFT-----PEGKSVNELSVPRILVAGGFAGIFN  223
D.rerio       -------AASGQ---VKYAGPMDCVKQLYR-ESGIRGIYKGTALTLMRDVPASGMYFMTYEWLKHALT-----PEGKSPSELSVPSILFAGGMAGIFN  222
C.elegans     -------QAGSAGSGVHYDGPLDVVKKLYK-QGGISSIYRGTGATLLRDIPASAAYLSVYEYLKKKFS-----GEG-AQRTLSPGATLMAGGLAGIAN  219
D.melanogaster -------QGQGGE--RKYNGMIDCAGKLYK-EGGLRSVRGSCATMLRDFAANGLYFLVYEALQDVAH-----SKS-ETGQISTASTIFAGGVAGMAY  228
A.thaliana    ----GASTTSSVVAAVKYGGPMDVARHVLRSEGGARGLFKGLFPTFAREVPGNATMFAAYEBAFKRFLA-----GGS-DTSSLGQGSLIMAGGVAGASF  227
O.sativa      PVGGGAATATASVAAVKYGGPVDVAKQVLRSEGGVGGLFKGLGPTLAREVPGNALMFGVYBAIKQYLA-----GGQ-DTSSLGRGSLVVAGGLAGASF  243
S.cerevisiae  ------KGS-----------FIQAAKTIVK-EGGIASLFKGSLATLARDGPGSALFASYEISKNYLNSRQPRQDAGKDEPVNILNVCLAGGIAGMSM  253
A.nidulans    ------QNPPPPGQKPKYSGGVDVVRQLYK-EGGIRSVFRGSAMTLARDGPGSAAYFAAYEYIKRSLTPKDANGNATGD--LSLPAVLAAGGAAGIAM  253

               h56           H6
              *   * *.:*: :*     .. : :    *    .:: *. . :   *:.***** *. *::     :
R.norvegicus  WVVAIPPDVLKSRFQTAP-PGKYPNGFRDVLRELIRE-EGVTSLYKGFNAVMIRAFPANAACFLGFEIAMKILNWI------APNL---------  301
M.musculus    WAVAIPPDVLKSRFQTAP-PGKYPNGFRDVLRELIRE-EGVTSLYKGFNAVMIRAFPANAACFLGFEIAMKFLNWA------APNL---------  301
H.sapiens     WAVAIPPDVLKSRFQTAP-PGKYPNGFRDVLRELIRD-EGVTSLYKGFNAVMIRAFPANAACFLGFEVAMKFLNWA------TPNL---------  301
B.taurus      WAVAIPPDVLKSRFQTAP-PGKYPNGFKDVLRELIRD-EGITSLYKGFNAVMIRAFPANAACFLGFEVAMKFLNWA------VPNL---------  301
D.rerio       WAVAIPPDVLKSRFQTAP-EGKYPNGFRDVLRELVRE-EGVRSLYKGFNAVMLRAFPANAACFLGFEVAMKFLNWI------APNL---------  300
C.elegans     WGVCIPADVLKSRLQTAP-EGKYPDGIRGVLREVIRE-EGPRALFKGFNAVFGLELTLAAFRYFGIGGHPTPSTEVVPLPHDE            312
D.melanogaster WILGMPADVLKSRLQSAP-EGTYKHGIRSVFKDLIVK-DGPLALYRGVTPIMLRAFPANAACFFGIELANKFFNIV------APNF---------  306
A.thaliana    WGIVYPTDVVKSVLQVDDYKNPRYTGSMDAFRKILKS-EGVKGLYKGFGPAMARSVPANAACFLAYEMTRSSLG--------------------  300
O.sativa      WGSVYPTDVVKSVLQVNPKYSGSMDAFRKILAS-EGVKGLYKGFGPAMARSVPANAACFLAYEVTRSALG--------------------  316
S.cerevisiae  WLAVFPIDTIKTKLQASS----TRQNMLSATKEIYLQRGGIKGFFPGLGPALLRSFPANAATFLGVEMTHSLFKKYGI-----------------  327
A.nidulans    WIPVFPVDTVKSRLQSAP----GKPTISGTIRTVYAA-GGFKAFFPGFGPALARAVPANAATFAGVELAHQFMNKLFD-----------------  326
```

Fig. 1. Alignment of proteins of the mitochondrial carnitine carrier subfamily. CAC proteins (NP446417 from *R. norvegicus*, NP000378 from *H. sapiens*, NP568670 from *A. thaliana*, NP014743 from *S. cerevisiae*, AJ011563 from *A. nidulans*, NP065266 from *M. musculus*, XP001253588 from *B. taurus*, NP957153 from *D. rerio*, NP501223 from *C. elegans*, NP477221 from *D. melanogaster*, and NP001065471 from *O. sativa*) were aligned using the Clustal W software. Identities are indicated by asterisks and conservative or highly conservative substitutions are indicated by dots or colons, respectively. The Lys, Arg, Glu and Asp residues, which are conserved and face the water-filled cavity according to the homology model of the human CAC (see Fig. 4), are highlighted in gray.

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

The previously constructed pMW7-WT human CAC recombinant plasmid [9] was used to introduce the mutations in the CAC protein. 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 using the Gene Clean Kit (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 expression vector. All mutations were verified by DNA sequencing and, except for the desired base changes, all the sequences were identical to that of human CAC cDNA. The resulting plasmids were transformed into *Escherichia coli* C0214. Bacterial overexpression, isolation of inclusion bodies, solubilization and purification of WT and mutant CAC proteins were performed as previously described [4].

2.3. Reconstitution of wild-type and mutant CAC proteins in liposomes

The recombinant proteins were reconstituted into liposomes as described previously [4,25]. The concentration of intraliposomal carnitine was 15 or 50 mM. The external substrate was removed from proteoliposomes on Sephadex G-75 columns.

2.4. Transport measurements

Transport was measured as [³H]carnitine/carnitine homoexchange. This is a suitable mode for measuring the activity of CAC in proteoliposomes [4,9,13,19,21,23], though it has no physiological relevance. Transport at 25 °C was started by adding 0.1 mM [³H] carnitine to proteoliposomes and terminated by the addition of 1.5 mM NEM [4,25]. In controls, the inhibitor was added together with the labeled substrate, according to the inhibitor stop method [25]. Finally, the external substrate was removed by chromatography on Sephadex G-50 columns and radioactivity in the liposomes was measured [25]. The experimental values were corrected by subtracting control values. Transport activities were calculated 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 [26] as previously described [4]. 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. [27], with the modifications reported in ref. [20]. The homology model of the human CAC was constructed using the structure of the bovine ADP/ATP carrier [18] as template and the computer application Swiss PDB Viewer [28] as previously described for the rat CAC [21].

3. Results

The role of the charged amino acid residues, which are conserved in the CAC subfamily (Fig. 1) and face the water-filled carrier cavity based on the CAC homology model (Fig. 5), has been investigated in the *Homo sapiens* recombinant protein by site-directed mutagenesis. The mutant proteins, over-expressed in *E. coli* and reconstituted in liposomes, were compared with the WT protein in terms of transport function and kinetic parameters of transport. The amount of single (Fig. 2 A and B) and double (Fig. 2C) mutant CAC proteins incorporated into liposomes was similar to that of the WT CAC. In
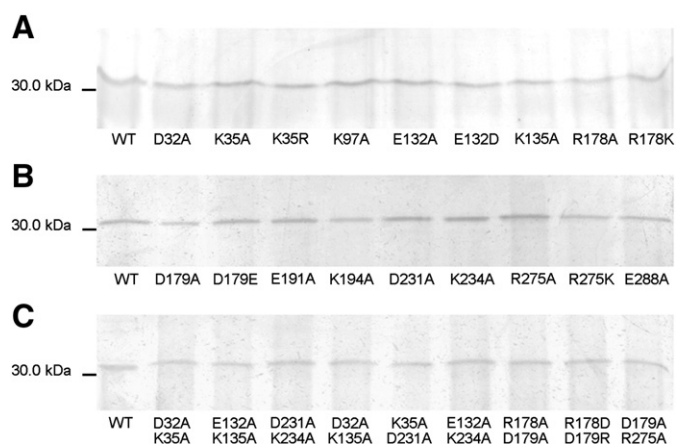

Fig. 2. WT and mutant CAC proteins incorporated into liposomes. Equal amounts of proteoliposomes reconstituted with WT and the indicated mutants of CAC were passed through Sephadex G-200 columns and 200 μl of the pass-through were ultracentrifuged at 110,000×*g*. The sediment was solubilized with 2% SDS, precipitated with acetone and then applied to the gel. After electrophoresis the gel was stained by silver-staining. The WT CAC was applied in the first line of each gel for comparison with single substitution mutants (A and B) and double substitution mutants (C).

Fig. 3 the transport activities of WT and mutants have been depicted as a function of time. Among the mutants of the positively charged amino acids Arg and Lys (Fig. 3A), K97A, K135A and K194A showed transport functions comparable to WT; at 30 min the transport activities of K97A and K135A were 88% of that of WT and the transport activity of K194A was 82% as compared to WT. R178A and K234A exhibited very low transport activities (about 15% of that of WT at 30 min), whereas K35A was nearly inactive (residual activity <7% of WT) as the previously described R275A mutant [9]. Among the mutants of negatively charged residues (Fig. 3B), E191A and E288A showed transport activities comparable to or somewhat lower than that of WT; at 30 min they were 103 and 70% of that of WT, respectively. The mutants D32A and D231A displayed much lower transport activities than that of WT; i.e., 36 and 14% at 30 min, respectively. E132A and D179A were nearly inactive with transport activities lower than 6% of WT.

To gain further insight into the role of the individual residues whose substitution with Ala led to nearly inactive proteins, conservative mutants were constructed. In Fig. 3C K35R, E132D, R178K and D179E showed transport activities of 37, 87, 38 and 49 %, respectively, compared to WT at 30 min. The amino acids Asp-32, Lys-35, Glu-132, Lys-135, Asp-231 and Lys-234 were also mutated with Cys, a residue which has been previously used in mutagenesis studies of other mitochondrial transporters [17,29–35]. As shown in Fig. 3C, the mutants K135C and K234C in contrast to the Ala mutants were nearly inactive, whereas the mutant D32C exhibited a transport activity (12% of WT at 30 min) lower than that of D32A shown in Fig. 3B. In addition, K35C, E132C and D231C were inactive (not shown). These results indicated that the electric charges of Lys-35, Glu-132, Arg-178 and Asp-179 are essential for transport and that the Cys residue is more deleterious to function than the Ala residue.
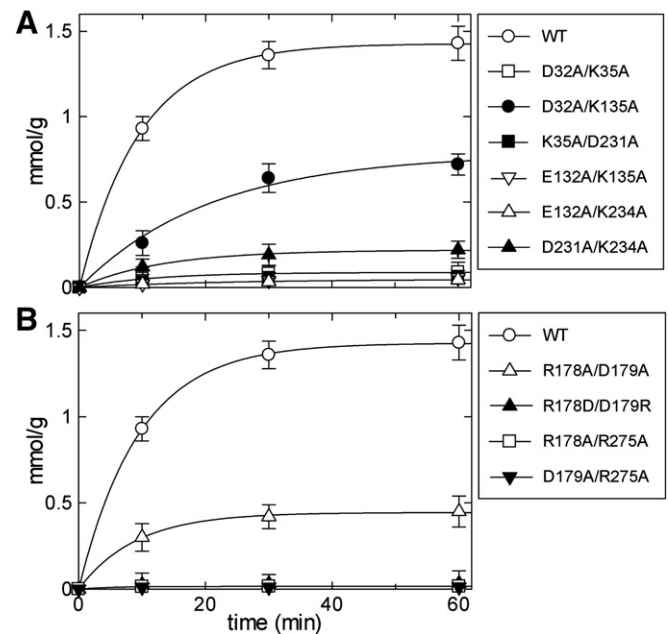
In another set of experiments, double mutants were constructed and assayed for transport activity in proteoliposomes. Fig. 4A shows the results of substitutions of amino acid pairs among the six charged residues of the carrier motifs (Fig. 6). Two types of double substitutions were performed; the first concerns residues located in different transmembrane segments (inter-helix) that are predicted to form the salt-bridge network closing the internal CAC cavity on the matrix side (D32A/K135A; K35A/D231A; E132A/K234A), and the second proximal residues located on the same transmembrane segment (intra-helix, D32A/K35A; E132A/K135A; D231A/K234A). Among the inter-helix double mutants (Fig. 4A), D32A/K135A was partially active with 41% of transport activity with respect to WT at
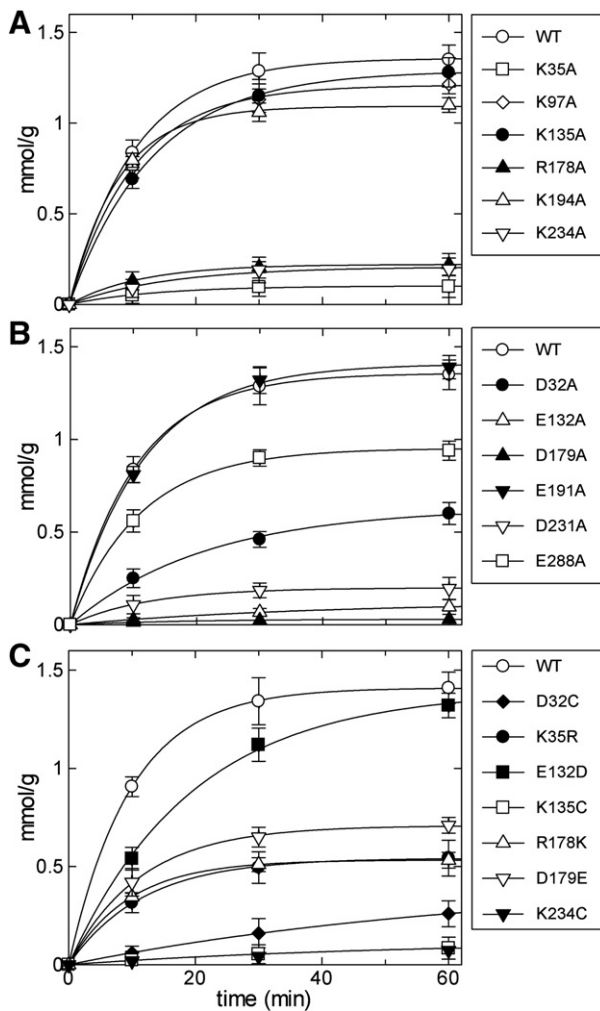
Fig. 3. Time course of [³H]-carnitine uptake in liposomes reconstituted with recombinant WT and single mutant CAC proteins. Transport was started by the addition of 0.1 mM [³H]-carnitine to proteoliposomes containing 15 mM carnitine and terminated at the indicated times. In each panel the data relative to the WT CAC (O) are shown together with those relative to the indicated CAC mutants. The data represent means ± SD of at least three independent experiments.

Fig. 4. Time course of [³H]-carnitine uptake in liposomes reconstituted with recombinant WT and double mutant CAC. Transport was started by the addition of 0.1 mM [³H]-carnitine to proteoliposomes containing 15 mM carnitine and terminated at the indicated times. In each panel the data relative to the WT CAC (O) are shown together with those relative to the indicated CAC mutants. The data represent means ± SD of at least three independent experiments.

largest variations in Km value (Table 2) were observed with the mutants K35A, E132A and D179E, which exhibited values more than three-fold greater than that of WT. Km values higher than that of WT, from 1.5 to 2.3 mM, were observed for the mutants K97A, K135A, R178A, K234A, and R275K. By contrast, the mutants D32A, K35R, E132D, R178K, E191A, K194A, D231A and E288A showed Km values similar to or not much lower than that of WT. Except for K97A, E132D, K135A, D179E and K194A, the other mutants showed significant decreases in Vmax as compared to WT. The relatively high SD of the

30 min, whereas the K35A/D231A and E132A/K234A double mutants were almost inactive with less than 7% transport activity compared to WT. The intra-helix double mutant D231A/K234A exhibited a transport activity of 15% compared to WT at 30 min, whereas the mutants D32A/K35A and E132A/K135A were nearly inactive with less than 7% of the function with respect to WT. Further combinations of double substitutions were performed on the three residues Arg-178, Asp-179 and Arg-275 found to be important for transport function (see Fig. 3 and ref. [9] for R275A activity). R178A/R275A and D179A/R275A exhibited transport activities lower than 5% of WT (Fig. 4B). In contrast, the R178A/D179A double mutant was much more active (31% of WT) than each of the single mutants R178A and D179A shown in Fig. 3. The double mutant R178D/D179R, in which the two adjacent amino acids were exchanged, was virtually as inactive as the double mutants R178A/R275A and D179A/R275A (Fig. 4B).

The effect of mutagenesis on the kinetic parameters of CAC is reported in Tables 1 and 2. In these experiments, the concentration of intraliposomal carnitine was increased from 15 to 50 mM to enhance the accumulation of the radioactive substrate inside the proteoliposomes, as was performed previously for the citrate carrier [32]. This increase in internal carnitine concentration did not significantly influence the Km of carnitine for the WT protein (not shown). The

Table 1
Kinetic constants of reconstituted CAC WT and single-replacement mutants.

Protein	Km (mM)	Vmax (μmol/min/mg)	Vmax/Km (ml/min/mg)
WT	0.86 ± 0.19	1.1 ± 0.30	1.3
D32A	0.68 ± 0.25	0.36 ± 0.13	0.53
K35A	2.8 ± 0.39	0.21 ± 0.09	0.08
K35R	0.95 ± 0.07	0.68 ± 0.29	0.72
K97A	1.5 ± 0.14	1.2 ± 0.42	0.80
E132A	3.0 ± 0.73	0.10 ± 0.06	0.03
E132D	0.86 ± 0.10	1.1 ± 0.26	1.3
K135A	2.0 ± 0.40	1.2 ± 0.60	0.60
R178A	2.0 ± 0.35	0.24 ± 0.07	0.12
R178K	0.81 ± 0.23	0.42 ± 0.08	0.52
D179A	n.m.	n.m.	
D179E	3.4 ± 0.47	0.90 ± 0.52	0.26
E191A	0.60 ± 0.15	0.56 ± 0.22	0.93
K194A	0.78 ± 0.16	0.90 ± 028	1.2
D231A	0.59 ± 0.20	0.19 ± 0.07	0.32
K234A	1.8 ± 0.51	0.44 ± 0.12	0.24
R275A[a]	n.m.	n.m.	
R275K[a]	2.3 ± 0.50	0.50 ± 0.19	0.21
E288A	0.62 ± 0.09	0.31 ± 0.07	0.50

The Km and Vmax values were calculated from double-reciprocal plots of the rates of carnitine/carnitine antiport under variation of the external substrate concentration. Transport was started by adding 0.12–2.0 mM [³H]carnitine to proteoliposomes reconstituted with CAC WT or one of the indicated mutants and containing 50 mM carnitine. The reaction time was 4 min. The data represent the means ± SD of four different experiments; n.m., not measurable; [a] data from ref. [9].

Table 2
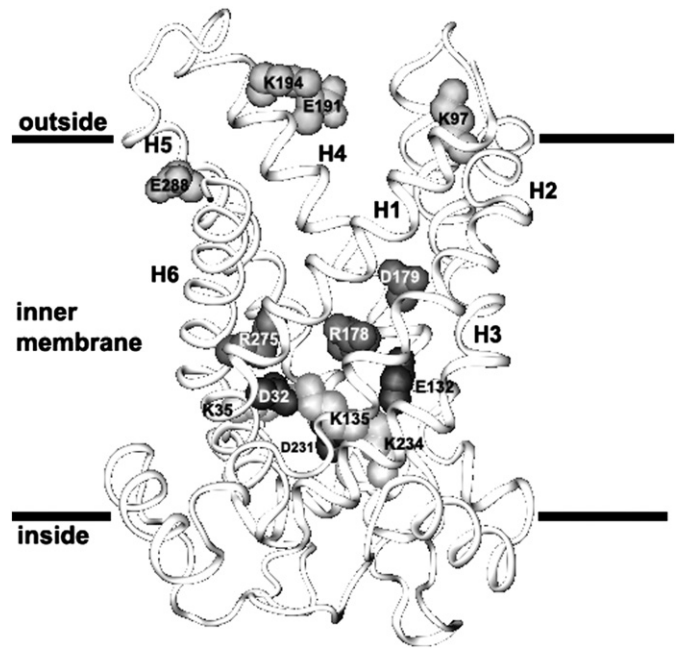Kinetic constants of reconstituted CAC double-replacement mutants.

Protein	Km (mM)	Vmax (µmol/min/mg)	Vmax/Km (ml/min/mg)
D32A/K35A	2.7 ± 0.80	0.10 ± 0.04	0.04
E132A/K135A	n.m.	n.m.	
D231A/K234A	1.7 ± 0.20	0.23 ± 0.18	0.14
D32A/K135A	2.5 ± 1.0	0.40 ± 0.24	0.16
K35A/D231A	2.9 ± 0.77	0.10 ± 0.04	0.03
E132A/K234A	n.m.	n.m.	
R178A/D179A	3.7 ± 0.64	0.36 ± 0.16	0.10
R178D/D179R	n.m.	n.m.	
D179A/R275A	n.m.	n.m.	

The Km and Vmax values were calculated from double-reciprocal plots of the rates of carnitine/carnitine antiport under variation of the external substrate concentration. Transport was started by adding 0.12–2.0 mM [³H]carnitine to proteoliposomes reconstituted with the indicated mutant and containing 50 mM carnitine. The reaction time was 4 min. The data represent the means ± SD of four different experiments; n.m., not measurable.

Vmax values is most likely due to variations in the amount of active protein within the different preparations. Most mutants displayed a decreased catalytic efficiency (Vmax/Km) compared to WT, with the exception of E132D and K194A. Furthermore, under the experimental conditions employed (i.e., internal carnitine concentration of 50 mM) the transport activities of D179A and R275A were still unmeasurable. Concerning the kinetic parameters of the CAC double mutants (Table 2), D32A/K35A, D32A/K135A, K35A/D231A and D231A/K234A exhibited Km values similar to those of the single mutants with higher Km reported in Table 2. On the other hand, with the exception of R178A/D179A, the Vmax values of the double mutants were comparable to those of the single mutants with lower Vmax reported in Table 2. Surprisingly, in the case of R178A/D179A, a Vmax of 0.36 µmol/min/mg protein was measured, although one of the single mutants (D179A) was completely inactive. In addition, the kinetic parameters of E132A/K135A, E132A/K234A, R178D/D179R and D179A/R275A could not be determined, since the transport activities of these proteins were unmeasurable even in the presence of 50 mM internal carnitine.
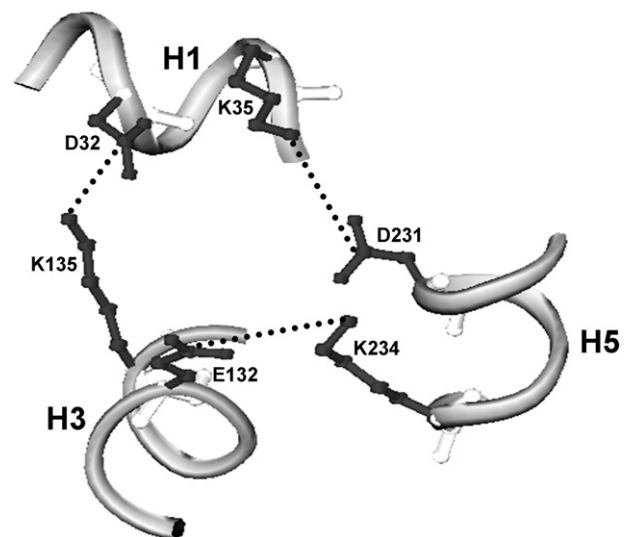
4. Discussion

The basic conformation of mitochondrial carriers in the c-state is a six α-helical bundle which delimits a water-filled cavity closed towards the matrix and opened towards the cytosol (see ref. [18] and Fig. 5). The substrate must enter the cavity and bind to specific residues to trigger the conformational changes required for opening the matrix gate and for substrate translocation. Carnitine has one positive and one negative charge; thus, it is expected to interact with both negatively and positively charged residues. Thus, we investigated the role of CAC charged residues that line the central water-filled cavity of the transport protein and are conserved in the CAC subfamily of mitochondrial carriers. These residues can be divided into three groups: (1) Asp-32, Lys-35, Glu-132, Lys-135, Asp-231 and Lys-234, which are located at the bottom of the cavity at the C-terminus of the odd-numbered transmembrane α-helices (Fig. 5, dark gray and white residues) and are predicted, on the basis of the structural similarity with the ADP/ATP carrier, to form an inter-helix salt-bridge network that closes the transporter in the c-state on the matrix side [18,36]. In CAC the three ion pairs would result from the interactions between Asp-32 and Lys-135, Glu-132 and Lys-234, and Asp-231 and Lys-35 (Fig. 6). (2) Arg-178, Asp-179 and Arg-275, which are located in the cavity at the midpoint of the membrane (Fig. 5, medium gray residues) and concern positions that have been proposed to constitute the common substrate binding site of mitochondrial carriers [37]. (3) Lys-97, Glu-191, Lys-194 and Glu-288, which are located at the C-terminus of the even-numbered transmembrane α-helices (Fig. 5, light gray residues) and have been proposed to form a salt-bridge network on the cytoplasmic side when the cavity is opened on the matrix side (m-state) [10]. In CAC the other two residues of the proposed network are the uncharged Gly-94 and Met-291.

The single replacements of Glu-132, Lys-234, Lys-35 and Asp-231 with Ala led to a strong reduction of transport activity (Fig. 3). By contrast, substitution of Asp-32 or Lys-135 with Ala maintains significant activity. Moreover, the Vmax value of the double mutant D32A/K135A is much higher than those of K35A/D231A and E132A/

Fig. 5. Structural model of CAC. Ribbon diagrams viewing the carrier from the lateral side. Dark gray (negatively charged residues) and white (positively charged residues) surfaces highlight the residues Asp-32, Lys-35, Glu-132, Lys-135, Asp-231 and Lys-234; medium gray surfaces highlight the residues Arg-178, Asp-179 and Arg-275; and light gray surfaces highlight the residues Lys-97, Glu-191, Lys-194 and Glu-288. The homology model has been represented using the molecular visualization program VMD.

Fig. 6. View of the CAC residues involved in the matrix salt-bridge network (matrix gate). The residues are depicted in a ball and stick representation with side-chains in dark gray and backbone in white. The salt bridges are highlighted by dotted lines. Segments of the odd α-helices H1, H3 and H5 containing the indicated charged residues are represented. The homology model has been represented using the molecular visualization program VMD.

K234A, the latter being too low to be determined. These results indicate that the ion pairs Glu-132–Lys-234 and Lys-35–Asp-231 (Fig. 6) are important for function, whereas residues Asp-32 and Lys-135 play a minor role. This conclusion parallels the observation that the matrix gate of several mitochondrial carriers contains one or even two weaker bonds than a salt bridge [3,10,30]. These weaker bonds are represented by a hydrogen bond (e.g., dicarboxylate and FAD carriers), a hydrophobic interaction (e.g., oxoglutarate and phosphate carriers) and an aromatic-hydrogen interaction (e.g., FAD, pyrimidine nucleotide and NAD$^+$ carriers).

The experimental data reported herein are consistent with the involvement of Arg-178, Asp-179 and Arg-275 in the binding of carnitine to the CAC protein, as hypothesized for the corresponding residues of the yeast CAC by bioinformatics [37]. Specifically, Asp-179 would interact with the carnitine trimethylammonium group whereas Arg-275 and Arg-178 with its carboxyl group. The Asp-179 and Arg-275 residues are fundamental for binding carnitine to CAC (Fig. 7A and B) since their replacement with Ala causes a complete loss of CAC activity. Furthermore, the electrical charge of the residues at positions 179 and 275 is more crucial for carrier function than side-chain length, since the substitution of either Asp with Glu at position 179 or of Arg with Lys at position 275 affects the Vmax value only slightly, as compared to that of the WT protein, whereas the Km value is markedly increased. A positive charge at position 178 is also important for CAC function. Thus, replacing Arg-178 with Lys does not significantly change the Km of carnitine for the carrier, whereas substituting Arg at this position with the neutral amino acid Ala causes a substantial increase in the Km. In addition, in both cases a significant decrease in Vmax occurs indicating that proper binding influences the rate of catalysis. We also propose that in the absence of transported substrate Arg-178 interacts with Asp-179 and this interaction causes a distortion of the adjacent residues and is easily broken by the oncoming substrate. This hypothesis is based on the observation that the complete loss of D179A mutant activity is partially rescued when a second mutation is introduced at position

178 leading to removal of the uncompensated positive charge, as in R178A/D179A. It is worth mentioning that in this double mutant the interaction of the carnitine trimethylammonium group with Asp-179 may be substituted, at least in part, by cation-π interactions [38,39] with Phe-86, Tyr-186 and Phe-187, which line the carrier cavity in the structural model of CAC.

The total binding energy of the interaction between the substrate and the transporter triggers the conformational changes that lead to the opening of the matrix gate and the closure of the cytosolic gate [3,10]. Although some crude hypotheses have been advanced [3,40], it is not yet known how the conformational changes occur and how the matrix gate opens. Also unknown is whether the substrate participates in gate opening, as hypothesized in ref. [40] and, for explaining the severe loss of activity caused by the R588Q mutation of the aspartate-glutamate carrier isoform 2 which is responsible for a disease named type II citrullinemia, in ref. [14]. The extremely low activities of the mutants K35A and E132A as well as their high Km values and very low catalytic efficiencies can be interpreted to indicate that the carboxyl and trimethylammonium groups of carnitine transiently interact with Lys-35 and Glu-132, respectively (not necessarily simultaneously), breaking the salt bridges Lys-35–Asp-231 and Glu-132–Lys-234 (Fig. 6). The importance of Asp-231 and Lys-234 in forming and breaking the matrix gate is in agreement with the substantial decrease in the Vmax values and catalytic efficiencies exhibited by the single mutants D231A and K234A. Moreover, it may be that the temporary interaction of carnitine with Lys-35 and/or Glu-132 is favored by the formation of a hydrogen bond between the carnitine hydroxyl group and His-29 (Fig. 7C) which contributes to the correct orientation of the substrate [23].

Among the charged residues predicted to be involved in the cytosolic salt-bridge network, the substitution of Lys-97 or Lys-194 with Ala is rather well tolerated. By contrast, replacement of E191 or E288 with Ala affects Vmax, indicating that these residues may play an important role in substrate transport. In light of the present results, the binding energy resulting from the interaction among the residues
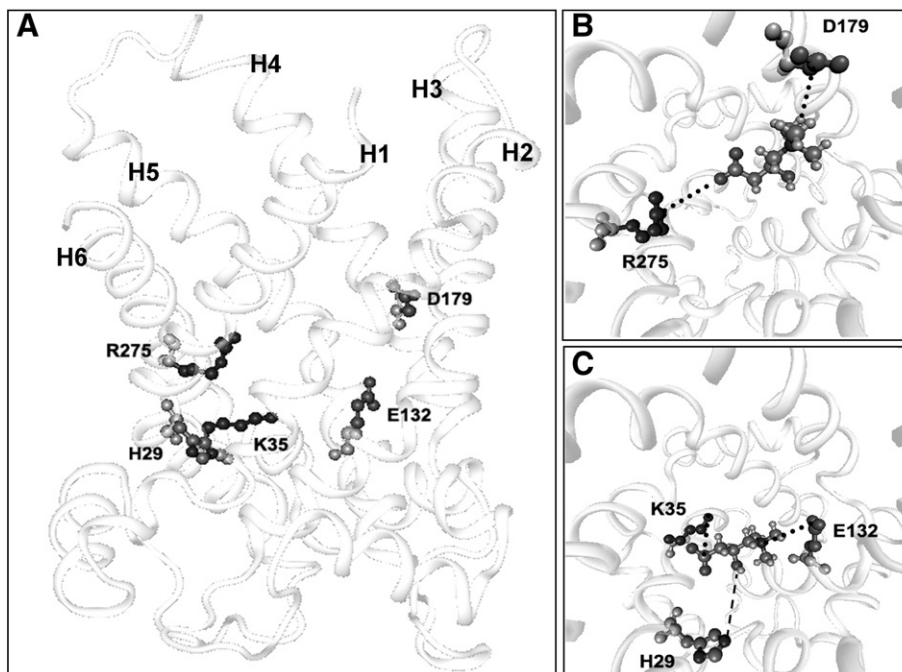
Fig. 7. View of the CAC residues which interact with carnitine. The residues and the carnitine molecule (in B and C) are depicted in a ball and stick representation. (A) Lateral view of the residues involved in interactions with carnitine. The backbones are light gray, the side-chains of the basic amino acids are dark gray and the side-chains of the acidic amino acids medium are gray; the side-chain of His-29 is depicted in medium gray except for the nitrogen atoms which are dark gray. (B) Top view, interaction of carnitine with D179 and R275. (C) Top view, interaction of carnitine with H29, K35 and E132. The ionic interactions are indicated by dotted lines and the hydrogen bond by a dashed line. The homology model has been represented using the molecular visualization program VMD. The position of carnitine and the interactions with the protein residues have been optimized using the software ArgusLab [41].

involved in the cytosolic salt-bridge network of CAC is predicted to be lower than that of other mitochondrial transporters. This would facilitate the conformational transition of the carrier from the m-state to the c-state in the absence of substrate, allowing CAC to catalyze the uniport mode of transport from the cytosol to the mitochondrial matrix [3,10] besides antiport, as previously demonstrated [12].

Acknowledgements

References

[1] C. Indiveri, V. Iacobazzi, N. Giangregorio, F. Palmieri, The mitochondrial carnitine carrier protein: cDNA cloning, primary structure, and comparison with other mitochondrial transport proteins, Biochem. J. 321 (1997) 713–719.
[2] F. Palmieri, The mitochondrial transporter family (SLC25): physiological and pathological implications, Pflugers Arch. - Eur. J. Physiol. 447 (2004) 689–709.
[3] F. Palmieri, C.L. Pierri, Structure and function of mitochondrial carriers—role of the transmembrane helix P and G residues in the gating and transport mechanism, FEBS Lett. 584, 1931–1939. Published online 25 October 2009.
[4] C. Indiveri, V. Iacobazzi, N. Giangregorio, F. Palmieri, Bacterial overexpression, purification, and reconstitution of the carnitine/acylcarnitine carrier from rat liver mitochondria, Biochim. Biophys. Res. Commun. 249 (1998) 589–594.
[5] M. Huizing, V. Iacobazzi, L. Ijlst, P. Savelkoul, W. Rutienbeek, L. van den Heuvel, C. Indiveri, J. Smeitink, F. Trijbels, R.J.A. Wanders, F. Palmieri, Cloning of the human carnitine-acylcarnitine carrier cDNA and identification of the molecular defect in a patient, Am. J. Hum. Genet. 61 (1997) 1239–1245.
[6] L. Palmieri, F.M. Lasorsa, V. Iacobazzi, M.J. Runswick, F. Palmieri, J.E. Walker, Identification of the mitochondrial carnitine carrier in *Saccharomyces cerevisiae*, FEBS Lett. 462 (1999) 472–476.
[7] J.R. De Lucas, A.I. Domínguez, S. Valenciano, G. Turner, F. Laborda, The acuH gene of *Aspergillus nidulans*, required for growth on acetate and long-chain fatty acids, encodes a putative homologue of the mammalian carnitine/acyl-carnitine carrier, Arch. Microbiol. 171 (1999) 386–396.
[8] S. Lawand, A.J. Dorne, D. Long, G. Coupland, R. Mache, P. Carol, Arabidopsis A BOUT DE SOUFFLE, which is homologous with mammalian carnitine acyl carrier, is required for postembryonic growth in the light, Plant Cell 14 (2002) 2161–2173.
[9] J.R. De Lucas, C. Indiveri, A. Tonazzi, P. Perez, N. Giangregorio, V. Iacobazzi, F. Palmieri, Functional characterization of residues within the carnitine/acylcarnitine translocase RX2PANAAXF distinct motif, Mol. Membr. Biol. 25 (2008) 152–163.
[10] A.J. Robinson, C. Overy, E.R. Kunji, The mechanism of transport by mitochondrial carriers based on analysis of symmetry, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 17766–17771.
[11] C. Indiveri, A. Tonazzi, F. Palmieri, Identification and purification of the carnitine carrier from rat liver mitochondria, Biochim. Biophys. Acta 1020 (1990) 81–86.
[12] C. Indiveri, A. Tonazzi, F. Palmieri, Characterization of the unidirectional transport of carnitine catalyzed by the reconstituted carnitine carrier from rat liver mitochondria, Biochim. Biophys. Acta 1069 (1991) 110–116.
[13] C. Indiveri, A. Tonazzi, F. Palmieri, The reconstituted carnitine carrier from rat liver mitochondria: evidence for a transport mechanism different from that of the other mitochondrial translocators, Biochim. Biophys. Acta 1189 (1994) 65–73.
[14] F. Palmieri, Diseases caused by defects of mitochondrial carriers: a review, Biochim. Biophys. Acta 1777 (2008) 564–578.
[15] E. Sekoguchi, N. Sato, A. Yasui, S. Fukada, Y. Nimura, H. Aburatani, K. Ikeda, A. Matsuura, A novel mitochondrial carnitine-acylcarnitine translocase induced by partial hepatectomy and fasting, J. Biol. Chem. 278 (2003) 38796–38802.
[16] J.A. Camacho, N. Rioseco-Camacho, The human and mouse SLC25A29 mitochondrial transporters rescue the deficient ornithine metabolism in fibroblasts of patients with the hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome, Pediatr. Res. 66 (2009) 35–41.
[17] A.R. Cappello, R. Curcio, D.V. Miniero, I. Stipani, A.J. Robinson, E.R. Kunji, F. Palmieri Functional, and structural role of amino acid residues in the even-numbered transmembrane alpha-helices of the bovine mitochondrial oxoglutarate carrier, J. Mol. Biol. 363 (2006) 51–62.
[18] E. Pebay-Peyroula, C. Dahout-Gonzalez, R. Kahn, V. Trezeguet, G.J. Lauquin, G. Brandolin, Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside, Nature 426 (2003) 39–44.
[19] C. Indiveri, A. Tonazzi, N. Giangregorio, F. Palmieri, Probing the active site of the reconstituted carnitine carrier from rat Liver mitochondria with sulphydryl reagents, Eur. J. Biochem. 228 (1995) 271–278.
[20] C. Indiveri, N. Giangregorio, V. Iacobazzi, F. Palmieri, Site-directed mutagenesis and chemical modification of the six native cysteine residues of the rat mitochondrial carnitine carrier: implications for the role of cysteine-136, Biochemistry 41 (2002) 8649–8656.
[21] A. Tonazzi, N. Giangregorio, C. Indiveri, F. Palmieri, Identification by site-directed mutagenesis and chemical modification of three vicinal cysteine residues in rat mitochondrial carnitine/acylcarnitine transporter, J. Biol. Chem. 280 (2005) 19607–19612.
[22] N. Giangregorio, A. Tonazzi, C. Indiveri, F. Palmieri, Conformation-dependent accessibility of Cys-136 and Cys-155 of the mitochondrial rat carnitine/acylcarnitine carrier to membrane-impermeable SH reagents, Biochim. Biophys. Acta 1767 (2007) 1331–1339.
[23] A. Tonazzi, N. Giangregorio, C. Indiveri, F. Palmieri, Site-directed mutagenesis of the His residues of the rat mitochondrial carnitine/acylcarnitine carrier: implications for the role of His-29 in the transport pathway, Biochim. Biophys. Acta 1787 (2009) 1009–1015.
[24] S.N. Ho, H.D. Hunt, R.M. Hortnon, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene 77 (1989) 51–59.
[25] F. Palmieri, C. Indiveri, F. Bisaccia, V. Iacobazzi, Mitochondrial metabolite carrier proteins: purification, reconstitution and transport studies, Methods Enzymol. 260 (1995) 349–369.
[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[27] A. Phelps, C. Briggs, L. Mincone, H. Wohlrab, Mitochondrial phosphate transport protein replacements of glutamic, aspartic, and histidine residues affect transport and protein conformation and point to a coupled proton transport path, Biochemistry 35 (1996) 10757–10762.
[28] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.
[29] V. Stipani, A.R. Cappello, L. Daddabbo, D. Natuzzi, D.V. Miniero, I. Stipani, F. Palmieri, The mitochondrial oxoglutarate carrier: cysteine-scanning mutagenesis of transmembrane domain IV and sensitivity of cys mutants to sulphydryl reagents, Biochemistry 40 (2001) 15805–15810.
[30] A.R. Cappello, D.V. Miniero, R. Curcio, A. Ludovico, L. Daddabbo, I. Stipani, A.J. Robinson, E.R.S. Kunji, F. Palmieri, Functional and structural role of amino acid residues in the odd-numbered transmembrane α-helices of the bovine mitochondrial oxoglutarate carrier, J. Mol. Biol. 369 (2007) 400–412.
[31] Y. Xu, A. Kakhniashvili, D.A. Gremse, D.O. Woods, J.A. Mayor, D.E. Walters, R.S. Kaplan, The yeast mitochondrial citrate transport protein, J. Biol. Chem. 275 (2000) 7117–7124.
[32] C. Ma, S. Remani, J. Sun, R. Kotaria, J.A. Mayor, D.E. Walters, R.S. Kaplan, Identification of the substrate binding sites within the yeast mitochondrial citrate transport protein, J. Biol. Chem. 282 (2007) 17210–17220.
[33] Y. Kihira, E. Majima, Y. Shinohara, H. Terada, Cysteine labeling studies detect conformational changes in region 106-132 of the mitochondrial ADP/ATP carrier of *Saccharomyces cerevisiae*, Biochemistry 44 (2005) 184–192.
[34] A. Iwahashi, Y. Kihira, E. Majima, H. Terada, N. Yamazaki, M. Kataoka, Y. Shinohara, The structure of the second cytosolic loop of the yeast mitochondrial ADP/ATP carrier AAC2 is dependent on the conformational state, Mitochondrion 6 (2006) 245–251.
[35] H. Wohlrab, The human mitochondrial transport protein family: identification and protein regions significant for transport function and substrate specificity, Biochim. Biophys. Acta 1709 (2005) 157–168.
[36] D.R. Nelson, C.M. Felix, J.M. Swanson Highly, conserved charge-pair networks in the mitochondrial carrier family, J. Mol. Biol. 277 (1998) 285–308.
[37] A.J. Robinson, E.R. Kunji, Mitochondrial carriers in the cytoplasmic state have a common substrate binding site, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2617–2622.
[38] D.A. Dougherty, Cation-π interactions in chemistry and biology: a new view of benzene, Phe, Tyr and Trp, Science 271 (1996) 163–167.
[39] X. Xiu, N.L. Puskar, J.A.P. Shanata, H.A. Lester, D.A. Dougherty, Nicotine binding to brain receptors requires a strong cation-p interaction, Nature 458 (2009) 534–537.
[40] E.R. Kunji, A.J. Robinson, The conserved substrate binding site of mitochondrial carriers, Biochim. Biophys. Acta 1757 (2006) 1237–1248.
[41] M.A. Thompson, ArgusLab 4.0.1 Planaria Software LLC, Seattle, WA (2004) http://www.arguslab.com.