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

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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.

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

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

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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 (h_{12}, h_{34} and h_{56}) 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-^{3}H]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.

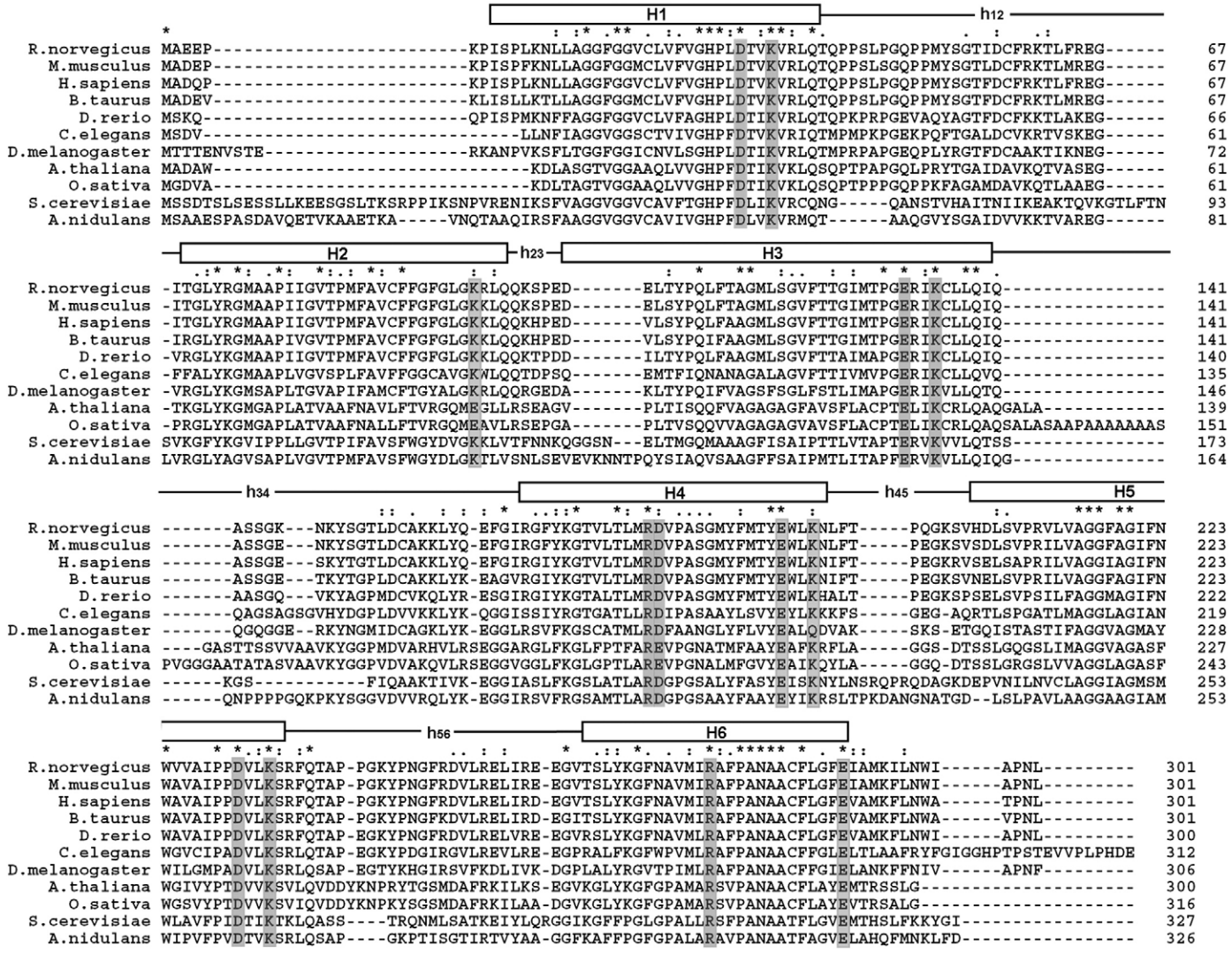

Fig. 1. Alignment of proteins of the mitochondrial carnitine carrier subfamily. CAC proteins (NP446417 from *R. norvegicus*, NP000378 from *H. sapiens*, NP568670 from *A. thaliana*, NP014743 from *S. cerevisiae*, AJ011563 from *A. nidulans*, NP065266 from *M. musculus*, XP001253588 from *B. taurus*, 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.