



## Review

## Structure and function of mitochondrial carriers – Role of the transmembrane helix P and G residues in the gating and transport mechanism

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## ABSTRACT

**To date, 22 mitochondrial carrier subfamilies have been functionally identified based on substrate specificity. Structural, functional and bioinformatics studies have pointed to the existence in the mitochondrial carrier superfamily of a substrate-binding site in the internal carrier cavity, of two salt-bridge networks or gates that close the cavity alternatively on the matrix or the cytosolic side of the membrane, and of conserved prolines and glycines in the transmembrane  $\alpha$ -helices. The significance of these properties in the structural changes occurring during the catalytic substrate translocation cycle are discussed within the context of a transport mechanism model. Most experimentally produced and disease-causing missense mutations concern carrier regions corresponding to the substrate-binding site, the two gates and the conserved prolines and glycines.**

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### 1. Introduction

Mitochondrial carriers (MCs) are nuclear-coded membrane proteins that transport a variety of solutes (di- and tricarboxylates, keto acids, amino acids, nucleotides and coenzymes/cofactors) across the inner mitochondrial membrane (see [1,2] for reviews). Besides providing a link between mitochondrial and other cell compartments, MCs play a role in regulating and maintaining a balance between cytosol and mitochondrial matrix, for example, of the phosphorylation and redox potentials. Some carriers, or one of their isoforms if any, are widely distributed in all or nearly all tissues, whereas others are tissue specific and their limited distribution reflects their importance in special tissues, e.g., for fatty acid biosynthesis or thermogenesis.

MCs are small proteins normally possessing a molecular mass of about 30–34 kDa. A main feature is the presence of three internal repeated sequences [3] which were noted immediately after the first MC, the ADP/ATP carrier, had been sequenced [4]. This striking pattern of repeats was also observed in the subsequent five MCs

sequenced, leading to the important conclusion that MCs belong to a single protein family, called the mitochondrial carrier family (MCF). All MCF members of known function exhibit a tripartite structure consisting of three tandemly repeated domains of about 100 amino acids in length. Each domain contains two hydrophobic stretches separated by extensive hydrophilic regions and a signature sequence motif PX[D/E]XX[K/R]X[K/R] (20–30 residues) [D/E]GXXXX[W/Y/F][K/R]G. Unlike the other members of the family, two subfamilies, the aspartate/glutamate and ATP-Mg/Pi carriers that share Ca<sup>2+</sup>-binding properties, have a peculiar structure consisting of two domains: a C-terminal domain or catalytic portion, which contains all the features of the MCF described above, and an N-terminal extensive domain or regulatory portion (more than 150 amino acids), which contains three or four EF-hand Ca<sup>2+</sup>-binding motifs. The substrates transported by MCs markedly vary not only in nature (anions, cations or zwitterions) and structure but also in size, from the smallest, H<sup>+</sup>, to the largest and most highly charged species transported through membranes, e.g., NAD<sup>+</sup>, FAD and coenzyme A. Moreover, the majority of MCs catalyze an obligatory 1:1 substrate exchange reaction. Only a few mediate unidirectional transport of substrates, such as carnitine, H<sup>+</sup>-compensated phosphate or H<sup>+</sup>-compensated glutamate, besides exchange.

This review highlights recent information and new insights about the structure and function of MCs. These novel concepts in the field of MCs, and in particular the role of the conserved prolines

*Abbreviations:* AGC1, aspartate/glutamate carrier isoform 1; MC, mitochondrial carrier; MCF, mitochondrial carrier family; OGC, oxoglutarate/malate carrier; Pi, phosphate; SLC25, human mitochondrial solute carrier family

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and glycines of the transmembrane  $\alpha$ -helices in the conformational changes occurring during the catalytic transport cycle, have been discussed within the context of a proposed mechanism model by which MCs operate.

## 2. Extension of the MCF

Until 1997, only six MCs were sequenced, after their purification from mitochondria, by Edman degradation or by molecular biology techniques based on minimal protein sequence information. They are the ADP/ATP carrier, the uncoupling protein and the carriers for phosphate (Pi), citrate, oxoglutarate/malate and carnitine/acylcarnitines (see [5] and references therein). In the post-genomic era, searching genome databases using the sequence features of the MCF has revealed a large number of family members. The genome of *Saccharomyces cerevisiae* encodes 35 MCs, that of *Arabidopsis thaliana* 58 and the human genome about 50. Actually, in man the MCF, named the human mitochondrial solute carrier family (SLC25), is by far the largest of the known solute carrier (SLC) families.

The first step in elucidating carrier function is to discover the substrate(s) transported by a particular carrier. One of the most effective methods to identify substrate specificity of new MCs consists of gene expression in *Escherichia coli* and/or *S. cerevisiae*, purification of the gene products and their reconstitution into liposomes in which substrate transport is assayed by direct measurements. By employing this strategy, numerous MCs have been identified at the molecular and biochemical level mainly in yeast, plants and humans (see [2,6–13] and references therein). Phylogenetic clustering, genetic information, knowledge of cell metabolism and complementation of phenotypes have often provided clues to the choice of substrate to be tested; yet, these data are not conclusive and substrate specificity remains to be determined by transport assays.

Based on their substrate specificity, MCs can be divided into subfamilies. Until now, the following subfamilies have been identified: uncoupler protein, and the carriers for ADP/ATP, Pi, 2-oxoglutarate/malate, citrate/malate, carnitine/acylcarnitines, dicarboxylates (malate/Pi), ornithine and other basic amino acids, succinate/fumarate, oxaloacetate/sulfate, oxodicarboxylates, adenine nucleotides in peroxisomes, aspartate/glutamate, glutamate, thiamine pyrophosphate and deoxynucleotides, S-adenosylmethionine, GTP/GDP, ATP-Mg/Pi, pyrimidine nucleotides, NAD<sup>+</sup>, coenzyme A/adenosine 3',5'-diphosphate, and FAD and folate (see [2,6,7] for reviews; [8–19]). Therefore, a total of 22 transport functions have been disclosed. Often, different subfamilies exhibit an overlapping of transported substrates, which also sometimes occurs for the defining substrates of each subfamily reported above. It is worth mentioning that the best substrate translocated in reconstituted liposomes might not be the most important substrate under physiological and pathological conditions and even in different tissues or specialized cells. Furthermore, some carriers may transport additional yet untested substrates, as has recently been found for  $\alpha$ -isopropylmalate as substrate of the yeast oxaloacetate/sulfate carrier, Oac1p [20]. In addition, most of the subfamilies reported above are present in all eukaryotes. However, a few subfamilies are more specific. For example, orthologs of the yeast GTP/GDP carrier are not present in mammals or plants [21] and therefore this carrier is a good target for novel drugs effective against pathogenic fungi and/or protista of *Homo sapiens* (e.g., *Candida albicans* and *Leishmania chagasi*) and plants (e.g., *Gibberella zeae*).

Further important steps in unravelling the physiological role of MCs are: their subcellular localization (as some MCF members were found in non-mitochondrial organelles, such as peroxisomes

and chloroplasts ([13] and references therein)); tissue distribution; kinetic parameters; metabolite distribution in different cell compartments; and phenotype analysis of organisms or cell lines in which a particular carrier gene had been knocked out/knocked down or overexpressed. This analysis has been commonly performed in yeast and to a lesser degree in both plants and animals.

## 3. Structure

According to the tripartite structure and hydrophobic profiles of the first sequenced carriers, it was suggested that each of the three repeats that make up MCs is folded into two membrane-spanning  $\alpha$ -helices forming a structure with a total of six  $\alpha$ -helices ([3,5] and references therein). This two-dimensional model of MCs was confirmed by topological data based on the accessibility of carriers to peptide-specific antibodies, proteolytic enzymes and other impermeable reagents. These data also proved that the N- and C-termini are exposed to the cytosolic side of the membrane, the loops connecting the two  $\alpha$ -helices of each repeat are located on the matrix side, and the two shorter segments connecting the three repeats are on the cytosolic side (see [22] for a review and references therein; [23]).

In 2003, the 3D structure of a member of the MCF, the ADP/ATP carrier in complex with its inhibitor, carboxyatractyloside, was determined [24]. This structure consists of a six-transmembrane  $\alpha$ -helix (H1–H6) bundle and three short  $\alpha$ -helices ( $h_{12}$ ,  $h_{34}$  and  $h_{56}$ ) parallel to the membrane plane on the matrix side. The six transmembrane  $\alpha$ -helices, in order from 1 to 6, line a funnel-shaped cavity open towards the cytosol in a counter-clockwise fashion. In each repeated domain, the first part of the signature motif, PX[D/E]XX[R/K], is located at the C-terminus of the odd-numbered transmembrane  $\alpha$ -helices, whereas the second part, [D/E]GXXXX[W/Y/F][R/K]G, is located at the N-terminus of the even-numbered transmembrane  $\alpha$ -helices. The prolines of the signature motif PX[D/E]XX[R/K] sharply kink the odd-numbered transmembrane  $\alpha$ -helices, and the charged residues of the three signature motifs form a salt-bridge network that closes the cavity (occupied by the inhibitor) on the matrix side. Among the possible considerations to be made about this crystal structure the following are worth mentioning: it should roughly correspond to the “c” (cytosolic)-state of the ADP/ATP carrier, as carboxyatractyloside is an inhibitor that blocks the carrier in this state; it exhibits a 3-fold pseudo-symmetry in line with the 3-fold sequence repeats, as also observed by electron microscopy of the yeast ADP/ATP carrier 2D crystals [25]; and, based on the strikingly conserved primary structure, it most likely represents the common folding of all the other MCF members and has therefore been used as a template for building homology models of various carriers. Furthermore, the ion pair network mentioned above constitutes the gate of MCs on the matrix side.

## 4. Findings based on sequence alignment analysis

Knowledge of substrate specificity of MCF members and the identification of subfamilies have been employed to analyze multiple sequence alignments of MCs on three different levels: complete sequences, sequences of the three repeats and sequences of the transmembrane  $\alpha$ -helices. Indeed, aside from the pioneering findings in 1982 of Saraste and Walker [3], remarkable and useful information has recently been obtained with this kind of analysis. By considering the amino acids conserved in alignments of carriers with known substrate specificity and using comparative models of these carriers, Robinson and Kunji [26] proposed a “common substrate-binding site” for MCs, i.e. a similarly located substrate binding site. This site is formed by the residues of the three

even-numbered transmembrane  $\alpha$ -helices that protrude into the cavity of the carrier at the midpoint of the membrane one-and-a-half helix turns above the matrix salt-bridge network that closes the bottom of the cavity. Among the proposed three contact points, point II on helix 4 is the most important because it alone can discriminate between amino acids, carboxylic acids (including keto acids) and nucleotides. In fact, point II is represented by R[D/E] in amino acid carriers, by R[QHNTAV] in carboxylic acid carriers and G[IVLMT] in nucleotide carriers. There are, however, some exceptions; for example, point II of ornithine carrier isoform 2 (ORC2) is QE and contact points II of Pi carrier and GTP/GDP carrier are [R/K]Q and RN, respectively, which are characteristic of carriers for carboxylic acids. Recent evidence indicates that the residues of the odd-numbered transmembrane  $\alpha$ -helices protruding into the cavity at the level of contact points I, II and III may also participate in substrate binding. In fact, the substrate of the yeast oxaloacetate carrier (Oac1p),  $\alpha$ -isopropylmalate, interacts with the side-chain of V37, which is the residue of the first transmembrane  $\alpha$ -helix (protruding into the cavity) at the height of the substrate-binding site [20]. Furthermore, some mutations of odd helix residues at the height of contact points (e.g., G133, A134 and S237 of the oxoglutarate carrier and H32 of the Pi carrier) inhibit transport activity [27,28].

Analysis of inter-repeat multiple sequence alignment of MCs of known function (substrate specificity) revealed triplets (formed by the aligned residues of each carrier), which may be either symmetrical, consisting of three identical amino acids, or asymmetrical, consisting of different amino acids [29]. Highly symmetrical and conserved triplets are important for structure and/or transport mechanism, whereas asymmetrical triplets for recognition and/or binding of the substrate (which usually has an asymmetrical distribution of functional groups). Based on the 3D structure of the ADP/ATP carrier, Fig. 1 shows the triplets of residues protruding into the cavity of *S. cerevisiae*, human and some *A. thaliana* carriers. These residues belong to the even-numbered (triplets 73–96) and odd-numbered (triplets 11–34) transmembrane  $\alpha$ -helices of the above carriers. The P (triplet 28) of the odd helices, and the G (triplet 73) and P (triplet 83) of the even helices are also shown in Fig. 1, though they do not protrude into the cavity. Typical asymmetrical triplets (80 and 81) correspond to the two amino acids which are located at contact points I, II and III. Typical symmetrical triplets (30 and 33) concern the positively and negatively charged residues involved in the matrix salt-bridge network [24] (formed by the charged residues of the sequence motif PX[D/E]XX[K/R]). Other symmetrical triplets, though less conserved (triplets 93 and 96), are localized at the C-terminus of the even-numbered transmembrane  $\alpha$ -helices and have been proposed to form a salt-bridge network on the cytosolic side (formed by the charged residues of the motif [F/Y][D/E]XX[R/K]) [29]. This ion pair network would close the carrier in the “m” (matrix)-state [29] and therefore constitute the gate of MCs on the cytosolic side. By considering the triplets of Fig. 1 in light of substrate specificity, it is apparent that MC subfamilies are characterized by specific triplets protruding into the carrier cavity. For example, the ATP-Mg/Pi carrier subfamily is characterized by triplets 84 (EYA) and 88 (KDS) and the thiamine pyrophosphate carrier subfamily by triplets 23 (R[T/S]K), 34 (IT[K/R]) and 85 (GAT). These triplets can therefore be used to predict the function (substrate specificity) of yet unidentified carriers. Nevertheless, (i) some of the characterizing triplets are shared by more than one subfamily, as for example triplet 19 in the NAD<sup>+</sup>, FAD and pyrimidine nucleotide carrier subfamilies, and triplets 22 and 85 in the glutamate and aspartate/glutamate carrier subfamilies, and (ii) various subfamilies are characterized by more than three triplets.

The inter-helix multiple sequence alignment of Fig. 2 (top panel) highlights (i) in the odd transmembrane helices the presence of a well-conserved glycine (orange arrow) nine residues before

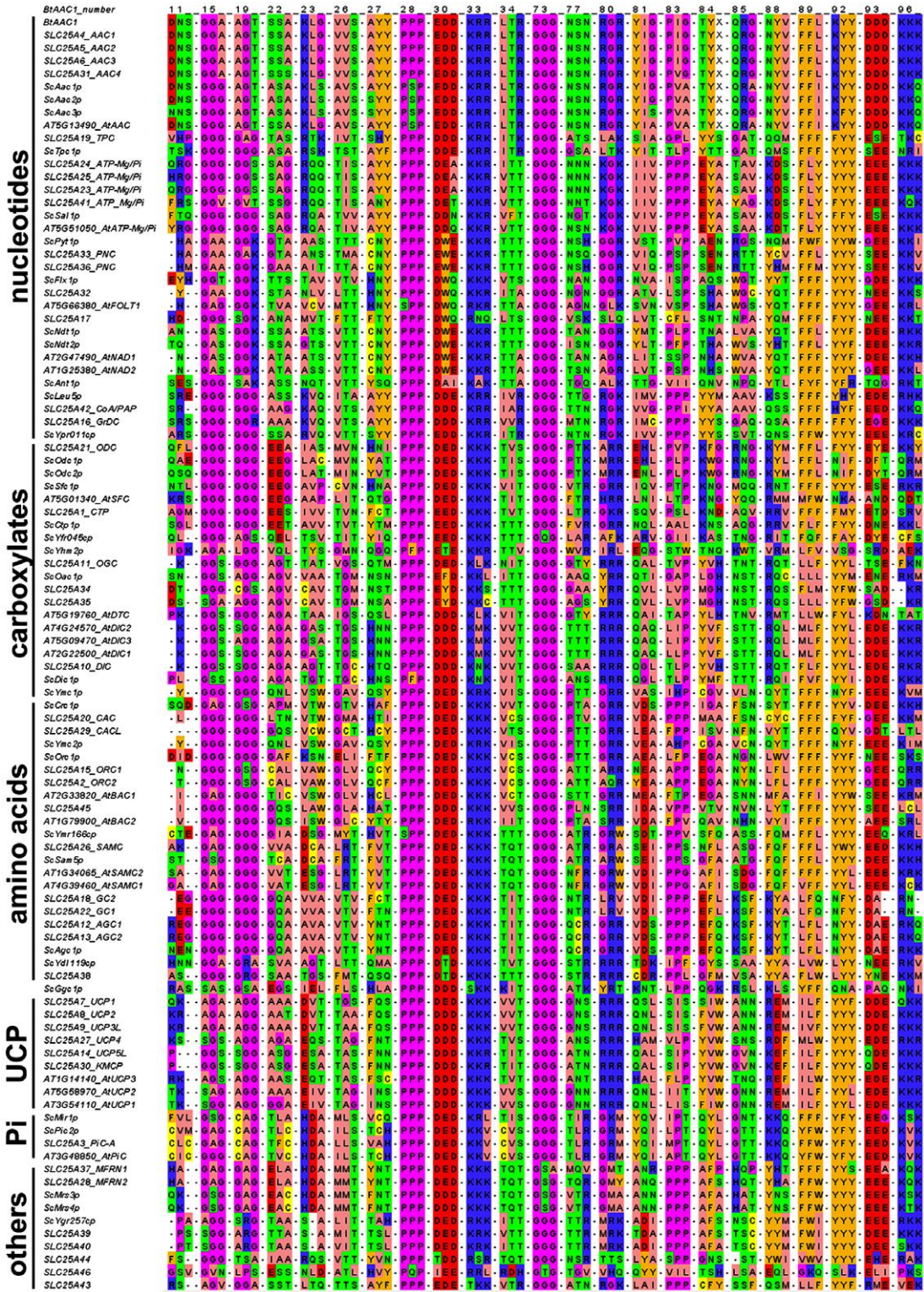
the prolines (grey arrow) of the PX[D/E]XX[K/R]X[K/R] sequence motif, and (ii) in the even transmembrane helices the presence of a conserved proline (grey arrow) 10 residues after the glycine (orange arrow) corresponding to the last residue of the second part ([D/E]GX[XX][W/Y/F][R/K]G) of the sequence motif. It is interesting that the above-mentioned G and P of the odd helices are aligned with the G and P of the even helices (Fig. 2, top panel), reflecting an antiparallel arrangement of odd and even helices (Fig. 2, bottom panel). The P of the odd helices and the G of the even helices are located about one-and-a-half helix turns below the substrate-binding site, and the G of the odd helices and the P of the even helices are about one helix turn above the substrate-binding site. Furthermore, the P of the odd helices are one-half helix turn above the matrix ion pair network or matrix gate, whereas the P of the even helices are two-and-a-half helix turns below the cytosolic ion pair network or cytosolic gate.

As proposed for the P of the odd helices [24], we hypothesize that the G of the odd helices and the G and P of the even helices can also act as hinges to open or close the carrier on the matrix or cytosolic side (see Section 6). It should also be noted that other rather conserved G are present in small regions immediately preceding the above-mentioned G in both the odd and even transmembrane helices (orange boxes in Fig. 2, top panel). The G of these regions may also participate in the hinge function of the highly conserved G. Moreover, in some carriers the P of the even helices are substituted by S, T and G (that can play a hinge role similar to that of P especially when other S, T or G are near in the sequence [30]) and less frequently by hydrophobic residues (which are known to be involved in helix formation and stabilization). In the latter case, however, we have noted that MCs usually display an incomplete cytosolic network. Interestingly, this is also true when P of the odd helices are rarely substituted with hydrophobic residues (e.g., in yeast dicarboxylate carrier [Dic1p], yeast suppressor of Hm mutant [Yhm2p] and SLC25A46).

## 5. Structure–function studies: the use of mutant proteins

The results of site-directed mutagenesis of the oxoglutarate carrier (OGC) and many other MCs [27,31–40] as well as the naturally occurring mutations of MCs found in patients (see [41] for a review), interpreted in the context of comparative models based on the 3D structure of the ADP/ATP carrier, can be summarized as follows:

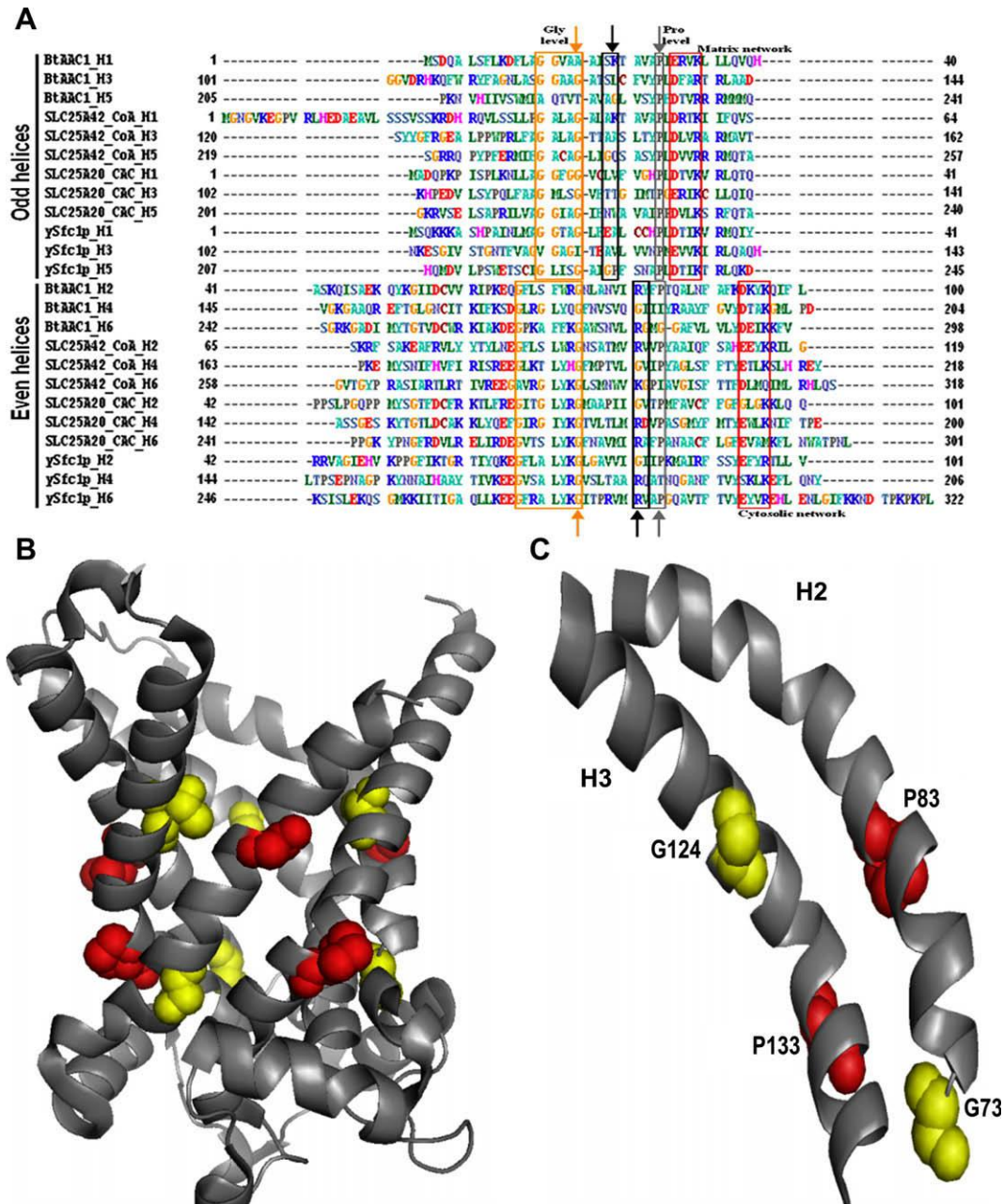
- (1) With a few exceptions, residues that interact with the lipid bilayer tolerate replacement with cysteine.
- (2) Few residues that participate in inter-helical interactions significantly inhibit transport activity when substituted. These residues may play a role in stabilizing the  $\alpha$ -helical bundle while the carriers undergo conformational changes.
- (3) Many residue replacements with cysteine located in the cavity of the carrier (or their subsequent modification by sulphydryl reagents) affect transport activity. In the case of OGC, among the 60 residues protruding into the cavity, 37 displayed significant transport inhibition when mutated to cysteine and 21 total inhibition ( $\leq 5\%$ ) as compared to the activity of C-less OGC. Critical for function are the charged residues at the bottom of the cavity, which belong to the signature motif PX[D/E]XX[K/R]. Also important for function are the residues of the even- and odd-numbered transmembrane  $\alpha$ -helices protruding into the cavity at the height of the substrate-binding site, because their substitution can interfere with binding. There are other residues protruding into the cavity that cannot be mutated into cysteine or modified by sulphydryl reagents without a significant loss in



**Fig. 1.** Alignment of amino acid triplets of 46 mitochondrial carriers (MCs) of *Homo sapiens*, 35 of *Saccharomyces cerevisiae* and some of their homologs in *Arabidopsis thaliana*. Each triplet is formed by the three aligned residues of each carrier, which are derived from the inter-repeat multiple sequence alignment of the MCs indicated above. The triplets are ordered horizontally according to the number of the first-repeat amino acids of the bovine ADP/ATP carrier sequence (NP\_777083). The carriers are listed according to the following major groups of substrates: nucleotides; carboxylic acids including keto acids; amino acids; and other substrates. Amino acids are coloured according to the default Jalview–Zappo style.

transport activity. These residues, located one, three and particularly two helix turns above the substrate-binding site and usually belonging more to the even than to the odd helices (according to available data), may also play a role in substrate recognition or binding.

(4) Also the importance of P and G helix residues is substantiated by site-directed mutagenesis. Thus, odd helix residues G32, G130, G230, P41, P139 and P239 as well as even helix residues G83, G87 and G183 (but not G281) mutated into cysteine severely affect OGC transport activity. In OGC the residues



**Fig. 2.** Prolines and glycines in the even and odd transmembrane  $\alpha$ -helices of MCs. Panel A: Inter-helix sequence alignment of selected MCs (AAC1, bovine ADP/ATP carrier isoform 1; CoA, human coenzyme A/adenosine 3',5'-diphosphate transporter; CAC, human carnitine/acylcarnitine carrier; Sfc1p, yeast succinate/fumarate carrier). Amino acids are coloured according to the default Bioedit style. Orange arrows indicate the conserved Gly in odd- and even-numbered helices; orange boxes highlight the Gly-rich regions in odd- and even-numbered helices; black arrows and boxes, the residues of the even and odd helices at the height of the common substrate-binding site; grey arrows and boxes, the conserved Pro in odd and even helices; and red boxes, the matrix and cytosolic salt-bridge networks. Panel B: Lateral view of the 3D structure of the ADP/ATP carrier (carboxyatractyloside not shown). The vertical triplet PPP of the odd helices (Panel A, top grey arrow) and the vertical triplet PIG of the even helices (Panel A, bottom grey arrow) are in red and in surf representation; the vertical triplet GGG of the even helices (Panel A, bottom orange arrow) and the vertical triplet AGT of the odd helices (Panel A, top orange arrow) are in yellow and in surf representation. Panel C: transmembrane  $\alpha$ -helices H2 and H3 taken from the crystal structure of the ADP/ATP carrier. H2 and H3 are depicted to show the position of a P (red) and a G (yellow) in each as well as helix curvature. The H2H3 helices were selected because they do not overlap.

corresponding to P, present 10 residues after the highly conserved G of the even helices, are T93, V193 and P291. Replacements of these residues with cysteine do not significantly inhibit activity. However, the OGC mutants of the adjacent residues Y94C and T293C exhibit transport of  $\leq 5\%$ .

## 6. Mechanism of transport

It is generally accepted that during the catalytic substrate translocation cycle, the carrier undergoes a reversible conformational

change between the c-state and the m-state. In the first case, the substrate-binding site is exposed toward the cytosol and, in the second, toward the mitochondrial matrix. The principal idea of this hypothesis is the presence of a single carrier-binding site (in the cavity) which is open alternatively to the two opposite sides of the membrane. According to this mechanism, named "single binding center-gating pore mechanism" [42], the binding of the substrate on one side induces structural changes in the carrier reorienting the binding site to the opposite side. The substrate now leaves the transport site allowing another substrate to be bound and transported in the opposite direction. This mechanism

was first suggested for the ADP/ATP carrier because of the existence of high-affinity inhibitor ligands that bind to the carrier either on the cytosolic side (atractyloside) or on the matrix side (bongkrekate). These ligands remove ADP or ATP and their binding is mutually exclusive, indicating a single reorienting binding site. The existence of a c-state has been demonstrated by the crystal structure of the carboxyatractyloside-ADP/ATP carrier complex [24]. In addition, the reorientation of the binding site is supported by the “common substrate binding site” [26] and by the kinetic “ping-pong mechanism” of carnitine/acylcarnitine carrier-catalyzed transport [43]. Unfortunately, a 3D structure of MCs in the m-state is not yet available. Moreover, the structural changes occurring during the reversible transition between the c- and m-states are unknown.

As an early event in substrate translocation through the carrier protein an uncharacterized substrate recognition may occur. Molecular dynamics studies indicate that a tyrosine ladder is important for sliding the ADP to the binding site [44,45], as originally suggested by Pebay-Peyroula et al. [24]. According to the “induced transition fit” theory [46,47], substrate binding in the cavity induces a rearrangement of the carrier in the transmembrane region to attain an optimum fit between the protein and the substrate corresponding to the transition state. Indeed, the carrier substrate-binding site is composed of all carrier cavity residues interacting with the substrate when the transition state is reached. It is proposed that in the transition state the carrier is nearly closed on both sides of the membrane and the substrate is bound at the center of the carrier (Fig. 3). In particular, the substrate interacts with the even and odd transmembrane helix residues of the similarly located binding site as well as with other residues protruding into the cavity above and below, depending on its size and shape. The total binding energy of the protein–substrate interactions in the transition state triggers additional structural changes which are necessary to complete the catalytic cycle, including the opening of the matrix or cytosolic gate (salt-bridge network) and the closure of the other gate on the opposite side of the membrane.

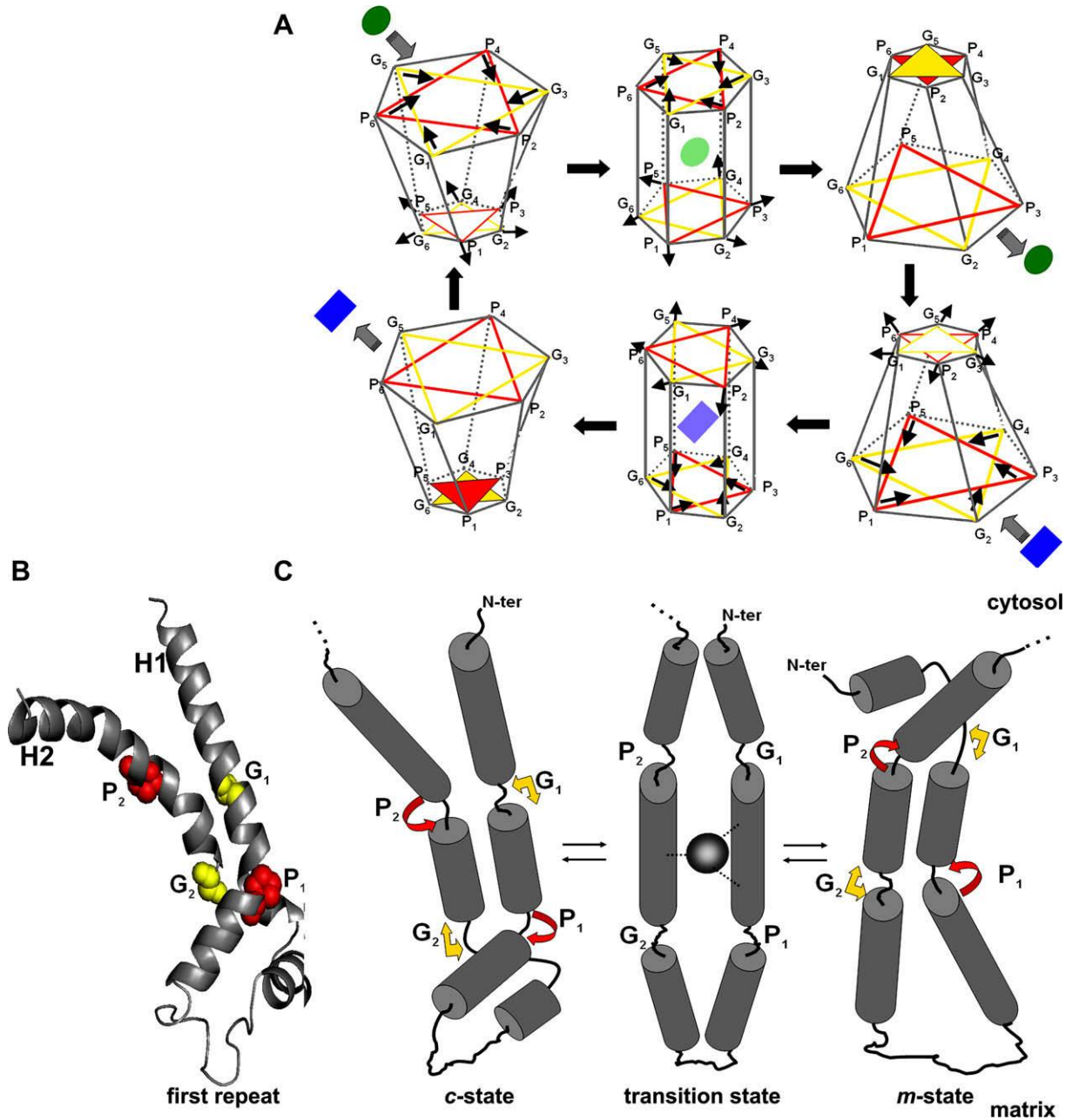
We propose a model illustrating the conformational changes involved in the transition between the two states based on current knowledge as well as the role of P and G of the transmembrane  $\alpha$ -helices discussed above. When the substrate enters the carrier in the c-state (see upper left truncated pyramid of Fig. 3A), as a result of the protein–substrate interaction helix 1 (defined by segment P<sub>1</sub>–G<sub>1</sub>) tilts towards helix 5 (P<sub>5</sub>–G<sub>5</sub>), helix 5 towards helix 3 (P<sub>3</sub>–G<sub>3</sub>) and helix 3 towards helix 1 (see arrows at the vertices of the yellow triangles of upper left truncated pyramid and top prism of Fig. 3A). The same occurs for even helices 2, 4 and 6 (see arrows at the vertices of the red triangles). When the substrate enters the carrier in the m-state (see lower right truncated pyramid of Fig. 3A), exactly the contrary occurs to complete the catalytic cycle (see arrows at the vertices of the red and yellow triangles of lower right truncated pyramid and bottom prism of Fig. 3A). For each transmembrane  $\alpha$ -helix, the tilt involves the entire helical segment including the termini above and below the P and G, which are not shown in Fig. 3A but are depicted in Fig. 3C. Looking at the carrier from the cytosol, even and odd helices would be seen moving clockwise during the c- to m-state transition, counter-clockwise during the m- to c-state transition, and vice versa looking at the carrier from the matrix side. Once the tilt is initiated by substrate binding to the c-state, a swivel of the P in the even helices occurs toward the cavity axis bringing together the [F/Y][D/E]XX[K/R] portions whose charged residues form the cytosolic salt-bridge network [29] and simultaneously the G in the odd helices act as hinges rotating their N-termini (towards the cavity axis) behind the cytosolic network (Fig. 3C, right side, upper segments). Conversely, after the tilt is induced by substrate binding to the m-state, the kink at the P in the odd helices reorients the PX[D/E]XX[K/R]

segments to form the matrix salt-bridge network, and a swivel of the G in even helices rotates their N-termini (towards the cavity axis) behind the matrix network (Fig. 3C, left side, lower segments), as seen in the crystal structure of the carboxyatractyloside-ADP/ATP carrier complex [24]. The 3D structure of the first repeat is shown in Fig. 3B for direct comparison with the schematic representation of a single repeat c-state depicted in Fig. 3C, left side. As a result of these structural changes, in the transition from the c- to the m-state, the gate on the matrix side breaks and the even–odd helices move apart while they come together to close the gate on the cytosolic side. The opposite occurs during the transition from the m- to the c-state. The model described above concerns the mechanism of MC-catalyzed substrate exchange. In the 1:1 exchange, the activation energy barrier for the reorientation of the substrate-binding site in the carrier is overcome only when the substrate is bound to the protein. For some carriers, which catalyze uniport (although at a lower rate) besides exchange, the activation energy barrier required for the conformational changes leading to the transition between the two states is much lower than that of obligatory exchange carriers [43,46]; thus, uniport carriers can reorient themselves in the absence of substrate. According to Robinson et al. [29], uniport mode of transport is related to the weakness of one or both salt-bridge networks. An additional consideration is that the above-described model implies that each MC monomer possesses an internal translocation channel or pathway. This means that MCs function as monomers, as strongly suggested by negative dominance studies conducted with the yeast ADP/ATP carrier [48].

## 7. Diseases

In the last decade, the completion of human nuclear gene sequencing and the ongoing identification of MC gene function enabled the discovery of numerous diseases associated to defective carriers (see [41] for a review and references therein). These disorders are caused by alterations of the genes encoding MCs, which are essential for either oxidative phosphorylation (e.g., the ADP/ATP and the Pi carriers) or other metabolic pathways and cell functions (e.g., the carriers for glutamate, aspartate/glutamate, thiamine pyrophosphate and carnitine/acylcarnitines). Their symptomatology depends on the specific metabolism affected and its relevance in specific tissues. To date, eleven MC-related diseases have been well characterized biochemically and genetically. With the exception of autosomal dominant progressive external ophthalmoplegia, all the other disorders are inherited in an autosomal recessive manner. The first nine diseases have been reviewed in 2008 [41]. Since then, two additional diseases, aspartate/glutamate carrier isoform 1 (AGC1) deficiency and congenital sideroblastic anemia, have been found.

AGC1 deficiency has been described in a child who presented with severe hypotonia, psychomotor developmental arrest and seizures beginning at a few months of age, followed later by spasticity [49]. At 8 months of age onward, magnetic resonance imaging of the brain revealed lack of myelination in the cerebral hemispheres, and proton magnetic resonance spectroscopy of the occipital and frontal lobes showed a drastically reduced peak of *N*-acetyl aspartate. The same traits were observed in knock-out mice for AGC1 [50]. Sequencing the patient's SLC25A12 gene revealed a homozygous c.1769A → G transition in exon 17, which produces a glutamine-to-arginine substitution at position 590 of AGC1 [49]. This mutation alters a highly conserved residue in the aspartate/glutamate MC subfamily, which protrudes into the internal cavity of the transporter just above the substrate-binding site and is deleterious for protein function. Indeed, recombinant mutant Q590R AGC1 reconstituted into liposomes was completely unable to cata-



**Fig. 3.** Schematic representation of conformational changes of the six MC transmembrane  $\alpha$ -helices occurring during the catalytic transport cycle. Panel A: Scheme showing the structural changes limited to the transmembrane helix segments between P and G. In this panel, the changes have purposely been exaggerated to aid the reader. This representation is intended mainly to emphasize the geometric perspective and, secondarily, to describe the transport mechanism. Truncated pyramids (left side) depict the c-state after the release (bottom) and immediately after the entry of the substrate (top); prisms indicate the transition states, and truncated pyramids on the right the m-state after the release (top) and immediately after the entry of the substrate (bottom). Substrate entering on the cytosolic side (green oval); substrate entering on the matrix side (blue rectangle). The six transmembrane  $\alpha$ -helices are designated by the segments that join the sides of prisms and truncated pyramids. The positions of the P and G of the even and odd helices are denoted by the vertices at the top and bottom bases of the geometric solids. In the bottom bases, the P of the odd-numbered helices are located at the vertices of the red triangles and the G of the even-numbered helices at the vertices of the yellow triangles. In the top bases, the P of the even helices are located at the vertices of the red triangles and the G of the odd helices at the vertices of the yellow triangles. The arrows along the sides of the triangles indicate tilt direction of the transmembrane helices. Panel B: Crystal structure of the bovine ADP/ATP carrier first repeat, taken from the published 3D structure of the carboxyatractyloside-ADP/ATP carrier complex. The conserved P and G positions in most MC odd and even transmembrane  $\alpha$ -helices (see Figs. 1 and 2A) are indicated by  $G_1$ ,  $P_1$ ,  $G_2$  and  $P_2$ . In the ADP/ATP carrier A19 is present instead of G. A19 ( $G_1$ ) and G73 ( $G_2$ ) are shown in yellow and in surf representation; P28 ( $P_1$ ) and P83 ( $P_2$ ) in red and surf representation. Panel C: Scheme showing the structural changes of the transmembrane  $\alpha$ -helices of a single repeat (including the segments external to the P and G residues, which are those that undergo major changes). For sake of clarity, the subscripts 1 and 2 were added to the P and G to more easily identify the odd- and even-numbered transmembrane helix. The left side corresponds to the c-state, the right side to the m-state and the middle part to the transition state; the grey oval in the transition state denotes the bound substrate; the yellow arrows denote the ability of G to bend the helices; and the red arrows indicate kink/swivel at the P. The angle of observation is the same as that of panel B. The carrier matrix axis is closer to the reader.

lyze transport of aspartate or glutamate, unlike wild-type AGC1. It was proposed that arginine in position 590 traps the substrate at the binding site impeding its movement through the protein and

interaction with the charged residues of the matrix gate [49]. AGC1 has two important functions: the first is to supply aspartate to the cytosol by catalyzing an exchange between intramitochondr-

drial aspartate and cytosolic glutamate [51], and the second is to participate in the transfer of reducing equivalents of NADH from cytosol to mitochondria being a key component of the malate-aspartate shuttle. Given that AGC1 is the only isoform of this carrier expressed in neurons, most symptoms of AGC1 deficiency are due to lack of AGC1 function in neurons. In brain, the aspartate exported from neuronal mitochondria is mainly utilized for the production of *N*-acetyl aspartate which, in healthy individuals, is the precursor of myelin lipids. Therefore, extensive and severe hypomyelination, the dominant feature of this syndrome, is caused by defective AGC1 in neurons. The symptomatology of AGC1 deficiency, on the other hand, suggests that the malate-aspartate shuttle is not so bioenergetically essential in neurons as generally thought, probably because the alternative pathway of reducing equivalent transfer into mitochondria, the glycerol-3-phosphate cycle, is sufficiently active in neurons. In AGC1 deficiency, impaired oxidative phosphorylation was ruled out based on the observations that lactate accumulation was minor or absent and no focal lesions in the basal ganglia and brainstem nuclei were revealed on magnetic resonance imaging. Moreover, mitochondrial ATP production was drastically reduced after incubation of the patient's muscle mitochondria with glutamate plus malate, but not with any other respiratory substrate tested [49].

Another error of metabolism is congenital sideroblastic anemia, which is caused by defects in the SLC25A38 gene [52]. As indicated by its name, this disease is characterized by severe anemia with hypochromia, microcytosis and ringed sideroblasts in the bone marrow (abnormal normoblasts with iron overload in the mitochondria). Patients are non-syndromic and present no developmental anomalies. Until now, 11 SLC25A38 alterations, including four missense mutations, have been found in patients in which X-linked ALAS-2 ( $\delta$ -aminolevulinic acid synthase-2) gene mutations were excluded [52]. In fact, ALAS-2, encoding erythroid-specific and intramitochondrially localized  $\delta$ -aminolevulinic acid synthase, is the only known gene, besides SLC25A38, to cause non-syndromic congenital sideroblastic anemia. The SLC25A38 gene is expressed in high amounts exclusively in erythroid cells, as ALAS-2, and encodes a yet uncharacterized MCF member that

here we call SLC25A38p. To elucidate the pathogenesis of altered SLC25A38-linked congenital sideroblastic anemia, elegant studies were conducted in zebrafish and *S. cerevisiae* demonstrating that SLC25A38p plays a role in heme biosynthesis [52]. This conclusion was based on the following observations: (i) silencing of the two zebrafish SLC25A38p homologs resulted in anemic embryos, although the anemia was not as severe as that caused by ALAS-2 silencing; and (ii) *S. cerevisiae* cells lacking the yeast homolog of SLC25A38p did not grow on the non-fermentable carbon source glycerol, were unable to reduce sodium nitroprusside (which requires heme-dependent cell-surface ferrireductase) and exhibited a markedly decreased amount of cellular  $\delta$ -aminolevulinic acid. Moreover, the SLC25A38-associated phenotype was similar to that caused by ALAS-2 mutations. In addition, the presence of an arginine-aspartate dipeptide in contact point II of SLC25A38p suggested that this protein is a carrier for amino acids. On this basis, it was hypothesized that SLC25A38p either imports glycine into mitochondria or exchanges cytosolic glycine for mitochondrial  $\delta$ -aminolevulinic acid across the mitochondrial membrane [52], i.e. transports one of the substrates and the product of the first step in heme biosynthesis catalyzed by ALAS-2, thus explaining the main feature of the disease, i.e. impaired heme production in erythroid cells.

Interestingly, among the 48 missense mutations found so far in patients affected by diseases associated to MCs (see [41] for a review; [49,52]), 30 concern residues that protrude into the internal carrier cavity. These 30 mutations are located in the following regions (Fig. 4): matrix gate (7); substrate-binding site (10); P-G level 1 (8); P-G level 2 (2); and cytosolic gate (3).

## 8. Conclusions

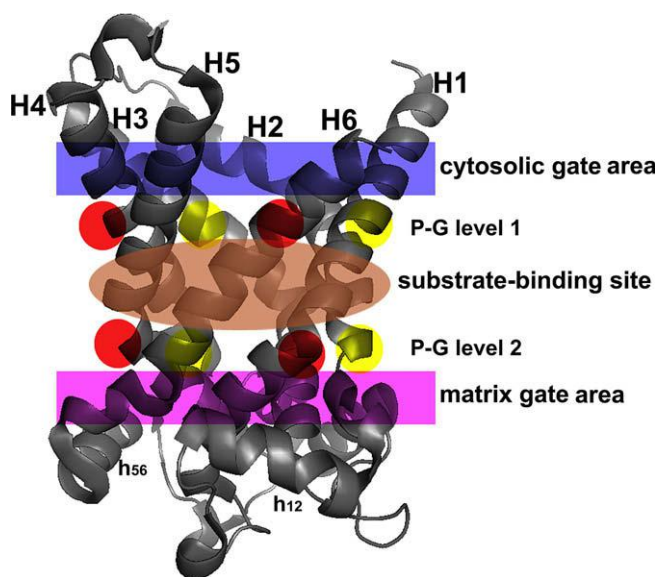
In recent years, significant progress has been made in characterizing MCs functionally and structurally. By utilizing a unique and powerful inhibitor, a breakthrough in the field occurred in 2003 when the 3D structure of the carboxyatractyloside-inhibited ADP/ATP carrier was made known [24] which, among other results, aided in interpreting many structural and functional data. This was followed by other exciting advances in understanding the functional mechanism of MCs, such as identification of the similarly located substrate-binding site based on knowledge of substrate specificity of carrier subfamilies, homology models and multiple sequence alignment analysis. However, much remains to be learned; most importantly, the precise structure of uninhibited carriers in different conformations. It is believed that the difficulties encountered in crystallizing MCs under the above conditions will be overcome in the future by improving the quality of proteins to be investigated.

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**Fig. 4.** MC structural areas in which many disease-causing missense mutations are located. The 3D crystal structure of the carboxyatractyloside-ADP/ATP carrier complex (devoid of the inhibitor) is shown with the following regions in colour: cytosolic gate (blue); substrate-binding site (orange); and matrix gate (purple). In P-G level 1 and P-G level 2, prolines are shown in red and glycines in yellow.



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