J Bioenerg Biomembr (2013) 45:1–13
DOI 10.1007/s10863-012-9475-7

MINI-REVIEW

The mitochondrial oxoglutarate carrier: from identification to mechanism

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Published online: 29 September 2012

Abstract The 2-oxoglutarate carrier (OGC) belongs to the mitochondrial carrier protein family whose members are responsible for the exchange of metabolites, cofactors and nucleotides between the cytoplasm and mitochondrial matrix. Initially, OGC was characterized by determining substrate specificity, kinetic parameters of transport, inhibitors and molecular probes that form covalent bonds with specific residues. It was shown that OGC specifically transports oxoglutarate and certain carboxylic acids. The substrate specificity combination of OGC is unique, although many of its substrates are also transported by other mitochondrial carriers. The abundant recombinant expression of bovine OGC in *Escherichia coli* and its ability to functionally reconstitute into proteoliposomes made it possible to deduce the individual contribution of each and every residue of OGC to the transport activity by a complete set of cys-scanning mutants. These studies give experimental support for a substrate binding site constituted by three major contact points on the even-numbered α-helices and identifies other residues as important for transport function through their crucial positions in the structure for conserved interactions and the conformational changes of the carrier during the transport cycle. The results of these investigations have led to utilize OGC as a model protein for understanding the transport mechanism of mitochondrial carriers.

Keywords Mitochondrial carrier · Oxoglutarate carrier · Membrane transporter · Transport mechanism · Substrate specificity

Abbreviations

AGC	Aspartate/glutamate carrier
CIC	Citrate carrier
MC	Mitochondrial carrier
MCF	Mitochondrial carrier family
OGC	Oxoglutarate carrier
PiC	Phosphate carrier

Introduction

The mitochondrial carrier family (MCF) comprises proteins responsible for the transport of metabolites, nucleotides and cofactors across the mitochondrial inner membrane, and in a few cases in other cellular membranes (Palmieri 2004; Palmieri 2012; Palmieri et al. 2011; Picault et al. 2004). Thereby the carriers connect enzymatic reactions belonging to metabolic pathways localized partly in the cytosol and partly in the mitochondrial matrix (or other compartments). For example, the ADP/ATP carrier exchanges cytosolic ADP for matrix ATP formed by the oxidative phosphorylation (Klingenberg 2008). Other carriers transport important specific solutes into and out of mitochondria, peroxisomes or chloroplasts (Agrimi et al. 2012; Palmieri et al. 2009, 2001b). MCs consist of six transmembrane α-helices that are equally distributed between three internal sequence repeats of about 100 amino acids each (Saraste and Walker 1982). Every repeat contains a signature motif with the

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sequence PX[DE]XX[RK] (PROSITE PS50920 and PFAM PF00153) by which MCF members are recognized.

One of the most well-characterized members of this family is the oxoglutarate carrier (OGC), also known as the oxoglutarate/malate antiporter, which plays an important role in the malate-aspartate shuttle and the oxoglutarate-citrate (isocitrate) shuttle. In this review, the aspects of OGC that have been studied over the past 40 years will be summarized. Early on this protein was identified, purified and sequenced. Then OGC became the first eukaryotic membrane protein to be expressed in *E. coli* and reconstituted in a functional state. This achievement opened up the field, made it possible to apply site-directed mutagenesis for detailed studies of carrier function and led to the rapid identification and characterization of other MCs. OGC has been the subject of intensive cysteine-scanning mutagenesis that has identified functionally important residues. Together with sequence analysis and the OGC homology model (based on the structure of the ADP/ATP carrier), this information has been used to deduce a proposed mechanism of MC transport.

Identification and characterization of OGC

A specific mitochondrial transport system for 2-oxoglutarate was first proposed by J.B. Chappell (Chappell 1968; Robinson and Chappell 1967) based on swelling experiments of isolated mitochondria in iso-osmotic solutions of ammonium salts of potential substrates. This was a very useful and indirect method to identify substrates that are translocated across the inner-mitochondrial membrane and suggest whether they are transported by symport with H^+ or by exchange. 2-Oxoglutarate transport was further characterized in isolated rat liver mitochondria (Palmieri et al. 1972), lending support to a carrier system capable of exchanging 2-oxoglutarate for malate. Oxoglutarate could be exchanged for L-malate, maleate, succinate and 2-oxoadipate; transport was inhibited by phenylsuccinate and butylmalonate, whereas no inhibition was observed with mono-, tri- and certain di-carboxylates. The kinetics of oxoglutarate and L-malate transport were characterized (Table 1), suggesting the existence of a single substrate binding site. The observed characteristics of oxoglutarate transport were confirmed also in rat heart and plant mitochondria (De Santis et al. 1976; Genchi et al. 1991; Sluse et al. 1973; Sluse and Liébecq 1973), while the yeast transport of oxoglutarate was thought to be different from that in mammalian systems (Perkins et al. 1973).

The protein responsible for oxoglutarate/malate antiport was first purified from pig heart mitochondria by solubilization in Triton X-114 and chromatography through hydroxyapatite and celite in the presence of cardiolipin (Bisaccia et al. 1985). The molecular weight of the protein was estimated to be approximately 32.5 kDa. Transport experiments with purified, reconstituted OGC demonstrated that the protein catalyzed electroneutral oxoglutarate exchange between oxoglutarate and malate. Malonate, succinate, oxaloacetate and maleate were also transported by the purified protein. The reconstitution procedure was optimized with OGC purified from bovine heart mitochondria (Indiveri et al. 1987b). The kinetic parameters were measured (Table 1), and the k_m for oxoglutarate was found to be similar to that measured in isolated mitochondria (Palmieri et al. 1972). In the 80s and beginning of the 90s the characterization of MCs was essentially based on their purification and reconstitution in phospholipid vesicles (Krämer and Palmieri 1989; Palmieri 1994; Palmieri et al. 1993a). In this respect, OGC was the fourth MC to be purified and reconstituted after the ADP/ATP carrier (Brandolin et al. 1980; Krämer et al. 1977; Krämer and Klingenberg 1977a, b; Lauquin et al. 1979), the uncoupling protein (Lin and Klingenberg 1980; Ricquier et al. 1982) and the phosphate carrier (PiC) (Bisaccia and Palmieri 1984; De Pinto et al. 1982; Kaplan et al. 1986). Following the purification of OGC, the dicarboxylate, carnitine, citrate, aspartate/glutamate, ornithine/citrulline and glutamine carriers were purified and functionally characterized (Bisaccia et al. 1988, 1989, 1990, 1992, 1996a; Capobianco et al. 1996; Dolce et al. 1996; Indiveri et al. 1990. 1991b, c, 1992a, b, 1994, 1995, 1997, 1998; Kaplan et al. 1990; Stipani et al. 1996; Zara et al. 1996). Kinetically the above-mentioned purified carriers, except carnitine/acylcarnitine, follow a sequential (simultaneous) mechanism of reaction (Bisaccia et al. 1993; Dierks and Krämer 1988; Indiveri et al. 1991a, 1993, 2001; Sluse et al. 1979; Stappen and Krämer 1994).

Table 1 Kinetic parameters determined for OGC 2-oxoglutarate homoexchange

	k_m (mM)	V_{max} (nmol/min/mg)	Reference
Rat liver mitochondria	0.046±0.0012[a]	42.8±2.2[a]	(Palmieri et al. 1972)
Purified and reconstituted from bovine heart	0.065±0.018[b]	4000–22000[b]	(Indiveri et al. 1987b)
Purified and reconstituted from bovine heat	0.31±0.08	8000–11000	(Indiveri et al. 1991a)
Reconstituted bovine OGC expressed in *E. coli*	0.2	2960	(Fiermonte et al. 1993)

[a] mg of total protein at 9 °C, pH=7.0

[b] at 25 °C, pH=7.0

When the sequence of the cDNA of bovine OGC became known (Runswick et al. 1990), it was clear that OGC belonged to the MCF, which at the time consisted of only the ADP/ATP carrier, the uncoupling protein and the PiC (Aquila et al. 1982, 1985; Runswick et al. 1987). The chromosomic DNA containing the human and bovine genes for OGC were sequenced and found to be comprised of 8 and 6 exons, respectively, spanning 2.5 or 2.3 kb of DNA, respectively, and encoding 314 amino acid long proteins with 96.6 % identical residues (Iacobazzi et al. 1992). Since the identification and sequencing of the human and bovine OGC genes many orthologs have been found exclusively in metazoa and plants. OGC is absent for example, in yeast, where oxoglutarate is transported by the oxodicarboxylate carrier (Palmieri et al. 2001a) and the citrate-oxoglutarate carrier (Castegna et al. 2010). Also, the MCF is now extended to more than 50 member sequences in man (Palmieri 2012; Palmieri et al. 2011), many of which have been discovered in the era of genomic sequencing.

In 1993, the bovine OGC gene was cloned and expressed in *E.coli* as inclusion bodies. The inclusion bodies were purified by centrifugation on sucrose gradients, solubilized in sarkosyl and reconstituted into liposomes in an active state (Fiermonte et al. 1993). The proteoliposomes loaded with various substrates were used in transport experiments where OGC worked as a strict antiporter exchanging the internal substrate with externally added radioactive 2-oxoglutarate. The previously determined substrate specificity was confirmed and the determined kinetic constants k_m and V_{max} were comparable to those previously obtained from purified OGC (Table 1). The heterologous expression of OGC in *E.coli* introduced a new method for producing MCs that were naturally expressed in low amounts and/or difficult to selectively purify for biochemical characterization. Since then, this recombinant expression technology has been used to produce sufficient amounts to functionally reconstitute and experimentally identify the substrates of many MCs (Palmieri 2004, 2012; Palmieri et al. 2006, 2011; Picault et al. 2004).

Substrate specificity and inhibitors of OGC

The recombinantly expressed and reconstituted OGC confirmed that the carrier transports 2-oxoglutarate, L-malate, succinate, oxaloacetate, malonate, maleate and, to a much lesser extent D-malate and 2-oxoadipate (Fiermonte et al. 1993). This finding denotes that OGC has overlapping substrate specificity with a number of other MCs; however, none of these transporters shares the complete set of OGC substrates (Table 2). The dicarboxylate carrier, for example, transports L-malate, malonate, maleate, succinate and oxaloacetate as does OGC but not any other 2-oxocarboxylates, and unlike OGC it also transports phosphate, sulfate and thiosulfate (Bisaccia et al. 1989; Fiermonte et al. 1998; Palmieri et al. 1996b). The oxodicarboxylate carriers transport oxaloacetate, 2-oxoglutarate and 2-oxoadipate, and the yeast forms also malate, as does OGC but these carriers also transport glutarate, adipate and 2-oxopimelate and not succinate and malonate (Fiermonte et al. 2001; Palmieri et al. 2001a). The yeast succinate-fumarate carrier (Sfc1p) shares substrate specificity with OGC for succinate, oxaloacetate and 2-oxoglutarate; unlike OGC it also transports fumarate but not malonate, maleate and malate (Palmieri et al. 1997). In addition, the oxodicarboxylate carriers as well as Sfc1p share low sequence identity with OGC, which implies that they are evolutionary distant and fulfill other functional roles than OGC. Another carrier for dicarboxylates is the yeast oxaloacetate carrier (Oac1p), which is substrate specific with OGC for malonate, malate and oxaloacetate but does not transport maleate, succinate, 2-oxoglutarate and 2-oxoadipate; in addition, Oac1p transports sulfate and α-isopropylmalate (Marobbio et al. 2008; Palmieri et al. 1999). The citrate carrier (CIC) overlaps with OGC by transporting the substrate malate and malonate (Bisaccia et al. 1988; Kaplan et al. 1990). In plants there is also a ditricarboxylate carrier that has the ability of transporting both OGC and CIC substrates: malate, oxaloacetate, oxoglutarate and citrate (Picault et al. 2002).

Most OGC inhibitors are substrate analogues like phenylsuccinate, butylmalonate, phtalonate and azido-phtalonate (Stipani et al. 1995), which are likely to bind in the binding site but not to be translocated and thereby block transport. Furthermore, all-trans-retinoic acid has an inhibitory effect on OGC (Cione et al. 2009). For the inhibition by mercurials and other reagents see “Structure of OGC”.

Physiological role of OGC

OGC plays an important role in several metabolic processes (Fig. 1), including the malate-aspartate shuttle which transfers the reducing equivalents of NADH from the cytosol into mitochondria. In the malate-aspartate shuttle 2-oxoglutarate and malate are transported by OGC, and aspartate and glutamate by the asparate/glutamate carrier (AGC). These substrates are converted by the mitochondrial and cytoplasmic forms of glutamate oxaloacetate transaminase and malate dehydrogenase. The net effect of the malate-aspartate shuttle is that that NADH in the cytosol is oxidized to NAD^+ and matrix NAD^+ is reduced to NADH. The role of OGC in the malate-aspartate shuttle was demonstrated in vitro by reconstitution of the purified OGC and AGC in proteoliposomes (Indiveri et al. 1987a).

Table 2 Substrate specificity of OGC and similar mitochondrial carriers

	MW	Oac1p (Palmieri et al. 1999)	Dicarboxylate carrier (Bisaccia et al. 1989; Fiermonte et al. 1998; Palmieri et al. 1996b)	OGC (Fiermonte et al. 1993)	Sfc1p (Palmieri et al. 1997)	Oxo-dicarboxylate carrier (Fiermonte et al. 2001; Palmieri et al. 2001a)	CIC (Kaplan et al. 1990)
Sulfate	92	X	X				
Phosphate	95		X				
Malonate	100	X	X	X			X
Maleate	112		X	X			
Fumarate	116				X		
Succinate	118		X	X	X		
Glutarate	132					X	
Oxaloacetate	132	X	X	X	X	X	
Malate	134	X	X	X		(X)	X
2-Oxoglutarate	144			X	X	X	
Adipate	146					X	
2-Oxoadipate	160			X		X	
2-Oxopimelate	172					X	
Cis-aconitate	174						X
Citrate	192						X

OGC is also involved in the oxoglutarate-isocitrate (citrate) shuttle, in which the reducing equivalents of NADPH are transferred from the mitochondria to the cytoplasm by transport of 2-oxoglutarate and malate by OGC, and isocitrate (citrate) and malate by the citrate carrier (see Fig. 3b in reference Palmieri 2004) (Hoek and Ernster 1974; Williamson and Cooper 1980). In the case of the oxoglutarate/isocitrate shuttle, the substrates are converted by the matrix and cytoplasmic forms of isocitrate dehydrogenase; in the case of oxoglutarate/citrate shuttle, oxoglutarate entering the mitochondria is converted into citrate by matrix isocitrate dehydrogenase and aconitase and citrate exiting the organelles by cytosolic citrate lyase and isocitrate dehydrogenase. Recently, the substrate specificity for

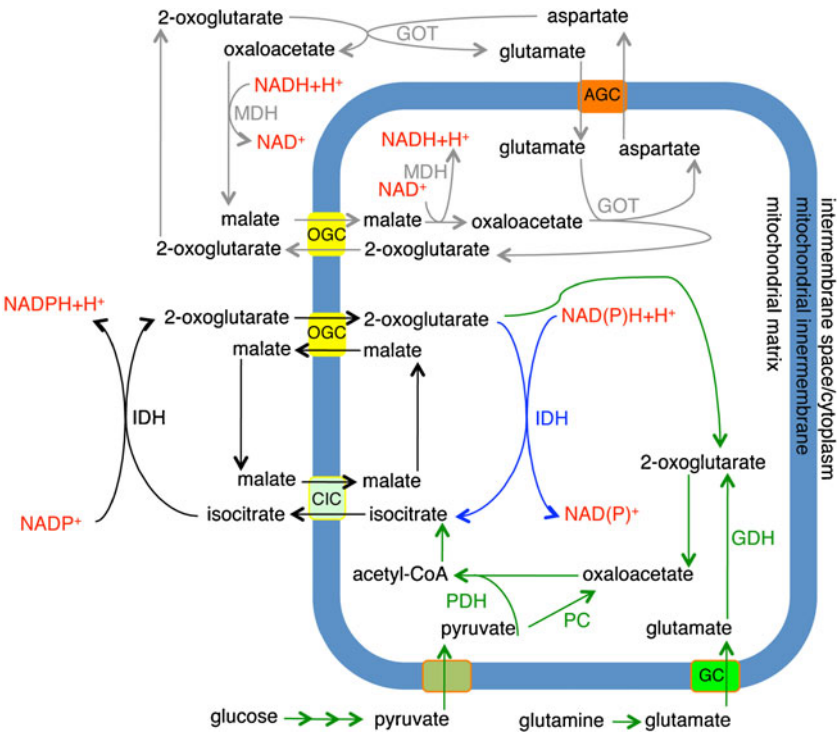

Fig. 1 Physiological role of OGC. The malate-aspartate shuttle (*grey arrows*), the oxoglutarate-isocitrate shuttle (*blue* and *black arrows*) and the pyruvate-isocitrate cycle (*green* and *black arrows*) are shown with the enzymes malate dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), pyruvate carboxylase (PC), pyruvate dehydrogenase (PDH) and glutamate dehydrogenase (GDH); and the carriers for oxoglutarate (OGC), citrate (CIC), aspartate/glutamate (AGC) and glutamate (GC)

yeast Yhm2p was determined. This protein is a citrate/2-oxoglutarate antiporter that in yeast could shuttle the exchange by itself (Castegna et al. 2010); however, no corresponding mammalian protein has been identified.

OGC has also been suggested to work in tandem with CIC in the pyruvate/isocitrate cycling pathway that is important for the regulation of glucose-stimulated secretion of insulin in pacreatic β-cells (Joseph et al. 2006; Odegaard et al. 2010). In this pathway mitochondrial pyruvate enters the citric acid cycle and citrate and/or isocitrate is exported by CIC to the cytosol where it is converted to 2-oxoglutarate and $NADP^+$ is reduced to NADPH by isocitrate dehydrogenase. 2-Oxoglutarate can then re-enter mitochondria via OGC.

Furthermore, OGC is believed to be involved in gluconeogenesis from lactate and nitrogen metabolism (Meijer and van Dam 1981). OGC together with the dicarboxylate carrier have also been suggested to transport glutathione (Chen et al. 2000; Coll et al. 2003; Lash 2006; Xu et al. 2006) although these findings remain controversial (Estrela et al. 2004). Porphyrin derivatives that are precursors for heme biosynthesis have also been suggested to be transported by OGC: however, these conclusions have been based on indirect experiments showing that these compounds bind and inhibit OGC and that oxoglutarate inhibits mitochondrial porphyrin transport (Kabe et al. 2006). Moreover, OGC has been implicated in uncoupling the mitochondrial membrane potential under certain experimental conditions (Yu et al. 2001). Finally, OGC has been suggested to play a role in mitochondrial fusion/fission, cell death and interact with regulators of apoptosis (Francia et al. 2004; Gallo et al. 2011).

It is noteworthy that there is also a chloroplast envelope 2-oxoglutarate/malate translocator involved in nitrogen metabolism in plants (Weber et al. 1995). However this transporter has 12 predicted transmembrane helices and does not belong to the MCF.

OGC biogenesis and localization

OGC has one single gene in bovine and human and in the latter the gene is located on chromosome 17 (Piccininni et al. 1998). The OGC gene is therefore nuclear-encoded; its mRNA is translated in the cytoplasm and the protein is targeted to mitochondria where it is translocated across the outer membrane and inserted into the inner membrane like all other MCs. The solubility and translocation competence of OGC in the cytoplasm is facilitated by the chaperones Hsc70 and Hsp90 (Zara et al. 2009). OGC and the majority of MCs lack an N-terminal mitochondrial targeting peptide and therefore have internal signals for mitochondrial localization. A few MCs, like the PiC and the CIC do have an N-terminal presequence that is proteolytically cleaved after import. However, these presequences are not necessary for import; they only assist import in some ways (Dietmeier et al. 1993; Zara et al. 1991, 1992, 2003, 2005, 2007). The targeting and insertion is dependent on ATP, the membrane potential and the mitochondrial outer membrane surface receptor Tom70, but not Tom20 (Palmisano et al. 1998). The insertion of MCs into the inner-mitochondrial membrane is mediated by the Tim22/54 complex. Evidence for an OGC topology with the N- and C-termini in the intermembrane space was obtained by antibodies directed towards these entities in bovine heart mitochondria and protease accessibility experiments on mitoplasts (Bisaccia et al. 1994). All mitochondrial carriers are thought to have the same topology as OGC (Capobianco et al. 1991; Capobianco et al. 1995, 1995; Palmieri et al. 1993b).

The tissue distribution of expressed OGC has been investigated. The rat OGC was found to be expressed in liver, heart and brain (Dolce et al. 1994), which is comparable with OGC expression in man. In the latter it is found in abundance in heart and skeletal muscle and in moderate amounts in brain, placenta, liver, kidney and pancreas, but absent in lung (Huizing et al. 1998). In the brain OGC is equally distributed between astrocytes and neurons, unlike AGC that is expressed preferentially in neurons and the glutamate carriers in astrocytes (Berkich et al. 2007).

Structure of OGC

Attempts were made to crystallize metal-ion chromatography-purified and reconstituted histidine-tagged OGC expressed in *E. coli* inclusion bodies (Smith 2003). However, the first structure of a MC was that of the native carboxyatractyloside-inhibited bovine ADP/ATP carrier solved by X-ray crystallography (Pebay-Peyroula et al. 2003). The structural model shows how the conserved signature sequence motifs come together in six transmembrane α-helices (H1-H6), forming a basket-like structure with the central cavity open towards the cytoplasmic side, and three additional α-helices on the matrix side (h_{12}, h_{34} and h_{56}). The prolines in the signature motifs introduce kinks in the odd-numbered α-helices that are stabilized by a network of inter-helical salt bridges between the charged residues of the signature motifs closing the bottom of the basket towards the matrix side. All MCs share the signature motifs and other sequence features of structural importance. Therefore, most likely all MCs have a fold similar to the structure of the bovine ADP/ATP carrier, at least in the conformation when the substrate binding cavity is open towards the cytoplasmic side. Thus, this structure has been used as a template in homology modeling for the other members of the MCF (Aluvila et al. 2010; Cappello et al. 2006; Giangregorio et al. 2010; Lawrence et al. 2011; Monné et al. 2012; Robinson and Kunji 2006).

In the case of OGC, several biophysical methods have been applied to characterize its structure and dynamics. Single transmembrane segments of OGC have been used in site-directed, spin-labeling experiments (Lauria et al. 2008; Morozzo della Rocca et al. 2003), circular dichroism and nuclear magnetic resonance in high concentrations of trifluoroethanol and sodium dodecyl sulfate (Castiglione-Morelli et al. 2004, 2005, 2007). Taken together the results of these experiments are in agreement with the accepted view that OGC is likely to have a structure similar to the ADP/ATP carrier.

Initial probing of the structure and function of OGC

Many of the previous biochemical studies where inhibitors and different molecular probes, that reacted with specific amino acid residues, were used to characterize OGC structure and function are coherent within the structural framework of OGC homology models (Fig. 2). Thiol reagents, such as mersalyl and p-chloromercuriphenylsulfonate, were shown to inhibit OGC suggesting that the protein contained cysteines at positions that were exposed and their modification lead to inactivated carrier (Bisaccia et al. 1985; Capobianco et al. 1996; Fiermonte et al. 1993). However, OGC was not inhibited by N-ethylmaleimide, which might suggest that this thiol reagent does not cause enough sterical hindrance to block transport. It was shown that bovine OGC could be inhibited by the membrane impermeable eosin-5-maleimide in intact mitochondria but not in submitochondrial particles, suggesting that it reacts with cysteines located towards the cytoplasm (Zara and Palmieri 1988). In the OGC homology model there are two cysteines located in a loop on the cytoplasmic side: C221 and C224 (Fig. 2). The last of the three cysteine residues in OGC, C184, exhibited more accessibility to sufhydryl reagents by adding substrate (Capobianco et al. 1996); this implies that the conformational changes induced by 2-oxoglutarate during the transport cycle expose this residue and that in the absence of substrate C184 is hidden in a percentage of the OGC molecules. Under special conditions the same cysteine has been shown to be able to cross-link two OGC molecules (Bisaccia et al. 1996b), which was interpreted as OGC being a dimer. In the structural model of OGC C184 is located at the beginning of H4 towards the matrix and could potentially form a disulfide bond with C184 of a neighboring OGC molecule. It is also likely that opening of the matrix gate involves conformational changes in the matrix helices or their repositioning, making the accessibility of C184 vary in accordance with substrate binding.

OGC is inhibited by pyridoxal 5′-phosphate, which indicates that at least one lysine residue is essential for substrate translocation and may be localized at or near the substrate binding site (Natuzzi et al. 1999). Similar experiments with probes reacting with arginine side chains suggested that at least one arginine residue is localized in or near the OGC substrate binding site (Stipani et al. 1996). In the structural model there are two lysines (K46 and K244) and four arginines (R90, R98, R190 and R288) close to the proposed substrate binding site (Fig. 2). However, there are many more positively charged residues facing the matrix and cytosolic sides, which could potentially block the entrance and exit of the substrate when they are modified.

Fluorescence spectroscopy has also been used in combination with homology modeling based to study substrate-induced conformational changes in OGC (Morozzo Della Rocca et al. 2005). In this study, a Trp-less OGC mutant and OGC mutants containing Trp instead of R190, C184 and L199 have been investigated in the presence or absence of oxoglutarate (Fig. 2). The substrate-specific quenching of the L199W mutant was shown to depend on the nearby residue K122, which when mutated into cysteine is more sensitive to inhibition of sulfhydryl reagents in the presence of substrate. These results indicate that these residues are located differently depending on substrate-induced conformational changes.

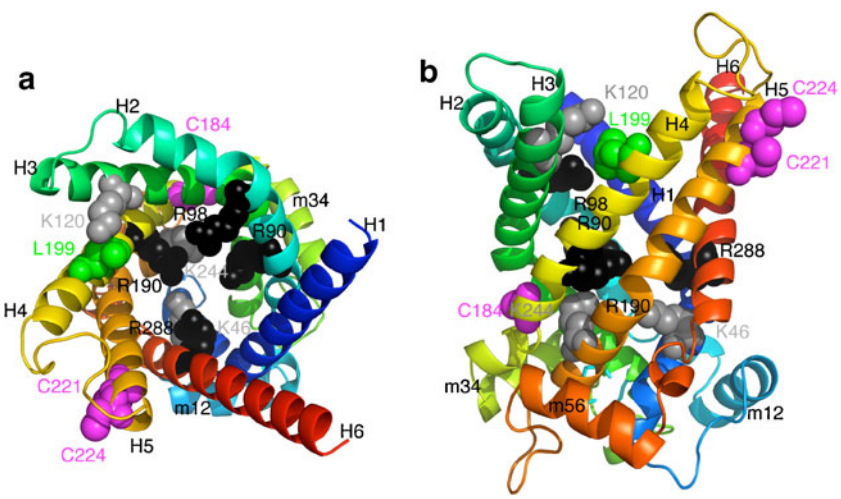

Fig. 2 Probed residues in OGC. The structural homology model of OGC is displayed in cartoon with rainbow colors from the N-terminal (*blue*) to the C-terminal (*red*) viewed from the cytoplasmic side (**a**) and laterally in the membrane plane (**b**). Arginine, lysine, cysteines and leucine residues discussed in the text are displayed as spheres colored black, grey, magenta and green, respectively

Functionally important residues and mechanism of OGC

Extensive cysteine-scanning mutagenesis assessed by transport experiments in the presence and absence of sulfhydryl-reactive agents has been performed in a Cys-less OGC construct (Palmieri et al. 1996a); the results have been evaluated within the structural framework of the OGC homology model (Cappello et al. 2006, 2007; Miniero et al. 2011; Stipani et al. 2001). Thus far, OGC is the only MC to have been investigated by complete cysteine-scanning mutagenesis. The identification of functionally important residues placed in the structural context renders the interpretation of their specific role more perceptive, increasing the understanding the functioning of OGC and perhaps other MCs. The large majority of cysteine replacement mutations that inhibit the transport function are found on the inside of the carrier cavity and around the so-called matrix gate that closes the bottom of the cavity (Fig. 3a–b). Very few of the residues that are in the loops and on the surface exposed towards the lipid bilayer are crucial for function. Most of the amino acids essential for activity belong to either of two groups: residues conserved in the OGC subfamily and residues that are conserved within entire MCF.

The first group of functionally important residues is found among the residues that are most likely involved in specific substrate binding. In the even-numbered helices, residues with side-chains protruding into the cavity at the midpoint of the membrane (R90, Y94 and R98 of H2, R190 of H4 and R288 of H6) were inactivated upon cysteine replacement, demonstrating their importance for OGC activity (Fig. 3c) (Cappello et al. 2006; Stipani et al. 2001). These residues are located in contact points I, II and III that

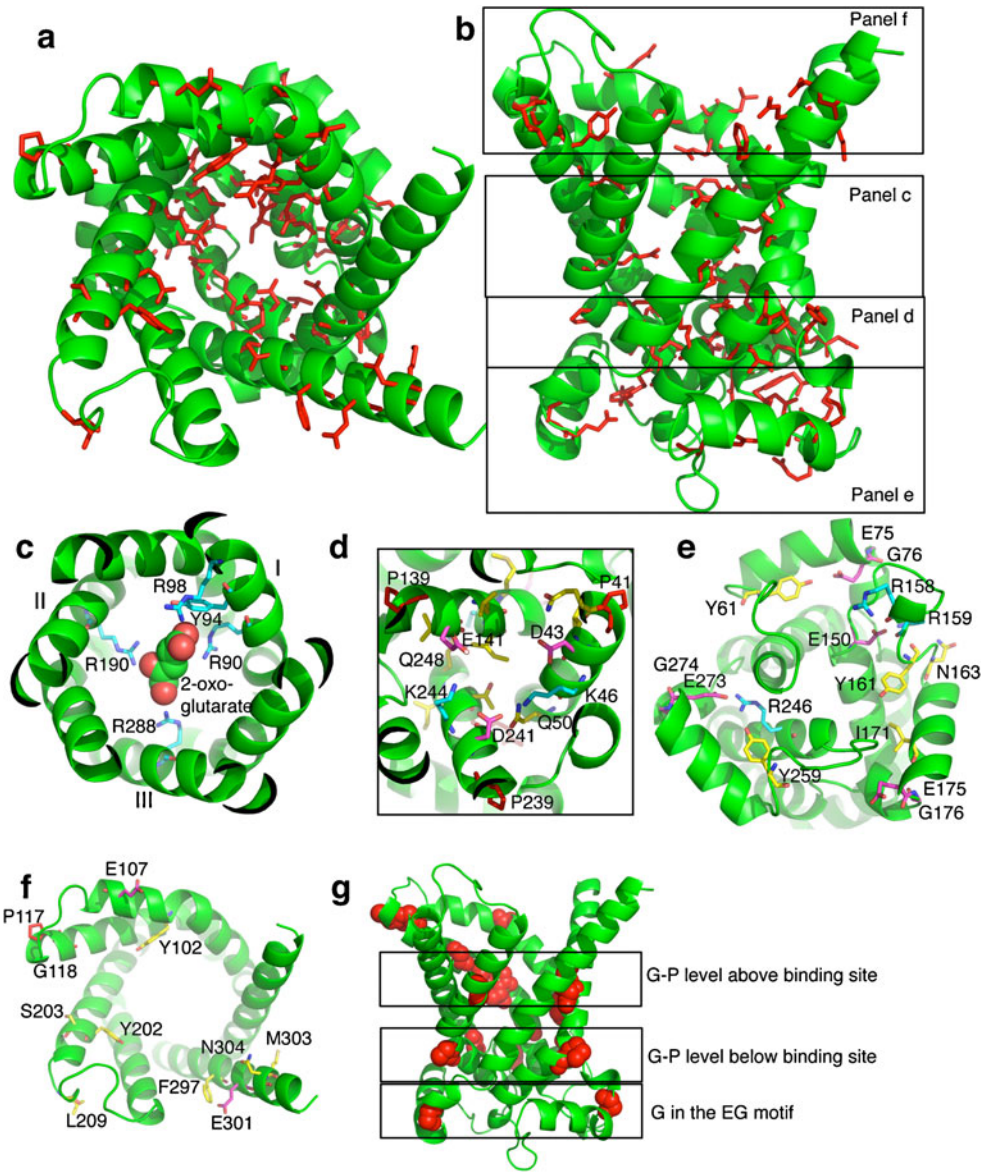

Fig. 3 Cysteine replacement mutations that inhibited OGC function. The OGC homology model is displayed as cartoon colored green with the residues that inhibited transport upon substitution with cysteine displayed as red sticks viewed from the cytoplasmic side (**a**) and laterally in the membrane plane (**b**) with indications of where the other panels are sliced. **c** The potential substrate binding site viewed from the cytoplasmic side with 2-oxoglutarte bound with only the residues shown to be functionally important in sticks. **d** The residues that have been shown to be crucial for OGC activity in the mitochondrial signature motif at the matrix gate viewed from the cytoplasmic side. Positively charged residues are colored cyan, negatively charged in magenta, proline in red and the other residues in yellow. **e** The essential residues of the matrix gate viewed from the matrix. **f** The functionally important residues of the cytoplasmic gate viewed from the cytoplasm. **g** The glycines and prolines that are crucial for OGC activity are shown as red spheres viewed laterally from the membrane plane

have been proposed to constitute the common substrate binding site of MCs (Robinson and Kunji 2006). The residues R90, R98, R190 and R288 had already been mutated into leucine and were found essential for transport (Palmieri et al. 1996a). Exactly how the residues in the binding site coordinate the different OGC substrates remains to be established. It is worth mentioning that mutations of the residues in the corresponding positions in other MCs have also been shown to inactivate transport function: R90 corresponds to yeast ADP/ATP carrier 2 AAC2-R96 (Heidkämper et al. 1996) and human uncoupling protein 1 UCP1-R83 (Echtay et al. 2001); Y94 to yeast citrate carrier CIC-K83 (Ma et al. 2007) and human ornithine carrier 1 ORC1-E77 (Monné et al. 2012); R98 to yeast phosphate carrier PiC-K90 (Briggs et al. 1999), CIC-R97 (Ma et al. 2007) and UCP1-R91 (Echtay et al. 2001); R190 to PiC-K179 (Briggs et al. 1999), CIC-R181 (Kaplan et al. 2000), ORC1-R179 (Monné et al. 2012), yeast carnitine/acylcarnitine carrier CAC-R178 (Giangregorio et al. 2010) and UCP1-R182 (Echtay et al. 2001); and R288 to CIC-R279 (Ma et al. 2007), CAC-R275 (Giangregorio et al. 2010), UCP1-R276 (Echtay et al. 2001), ORC1-R275 (Monné et al. 2012) and the human folate carrier-R288 (Lawrence et al. 2011). The results suggest that the loss of activity in these mutants is caused by a disrupted substrate binding site.

The second group of residues that are important for OGC function are found in the conserved sequence motifs. These residues are most likely important for the structural integrity of MCs and the dynamics of the transport cycle. The conserved residues in the PX[DE]XX[RK] signature motifs of the odd-numbered helices are crucial for OGC function (Fig. 3d) (Cappello et al. 2007), probably because they are involved in the salt bridge network forming the matrix gate in the conformational state of the carrier when the translocation pore is closed towards the matrix side. In OGC, these residues are P41, D43 and K46 of H1; P139 and E141 of H3 (where the positively charged residue is not conserved); and P239, D241 and K244 of H5. The basic residue in H3 in OGC is substituted by a leucine probably forming hydrophobic interactions with I145 and L51 (Cappello et al. 2007). In addition, there are several residues around the matrix salt bridge network that are important for function. For example, below the two salt bridges in OGC the conserved glutamine in the extension of the signature motif sequence PX[DE]XX[RK]X[RK]XQ is also essential for transport activity (Cappello et al. 2007). These results are supported by mutation studies in other MCs where the corresponding positions have been mutated leading to loss of transport activity: D43 corresponds to PiC-D39 (Briggs et al. 1999); K46 to AAC2-K48 (Nelson et al. 1998), PiC-K42 (Briggs et al. 1999) and CIC-K37 (Ma et al. 2007); E141 to AAC2-D149 (Nelson et al. 1998), PiC-E137 (Phelps et al. 1996), CIC-E131 (Kaplan et al. 2000) and CAC-E132 (Giangregorio et al. 2010); D241 to AAC2-D249 (Nelson et al. 1998), PiC-D236 (Phelps et al. 1996) and CAC-D231 (Giangregorio et al. 2010); and K244 to AAC2-K252 (Heidkämper et al. 1996), CIC-K237 (Ma et al. 2007), CAC-K234 (Giangregorio et al. 2010) and the folate carrier-R249 (Lawrence et al. 2011).

On the matrix side of the matrix gate essential residues are located around the conserved and symmetric residues Y and [ED]G at the beginning and the end of the matrix helices, respectively. They take part in interactions with residues in the tilted odd-numbered helices below the PX[DE]XX[RK] signature motifs (Fig. 3e) (Miniero et al. 2011). In OGC the conserved matrix helix residues are Y61, E75 and G76 of h_{12}; Y161, E175 and G176 of h_{34}; and Y259, E273 and G274 of h_{56}. A salt bridge is formed between E273 of h_{56} and R246 that is located two residues below the PX[DE]XX[RK] signature motif of H5, a position that is occupied by a positively charged residue in many MCF members including the ADP/ATP carrier. This finding suggests that in those carriers these residues form another conserved salt bridge network stabilizing the closed matrix gate on the matrix side. Unfortunately, there are no mutant studies of the corresponding positions in other MCs reported.

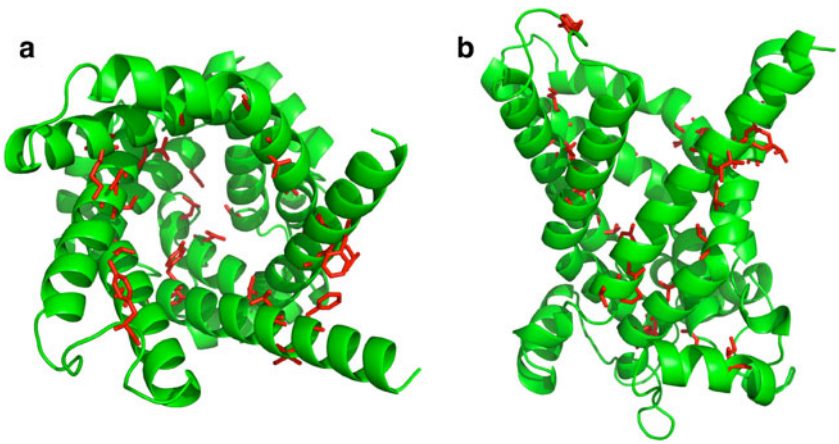

Fig. 4 Cysteine replacement mutations that inhibited OGC function when modified by sulfhydryl reagents. The OGC homology model is displayed as cartoon colored green viewed from the cytoplasmic side (**a**) and laterally in the membrane plane (**b**) with the residues that inhibited transport upon substitution with cysteine and modified with sulfhydryl reagents displayed as red sticks

On the cytoplasmic side of MCs the symmetric [DE]XX[RK] motif localized at the C-terminal end of the three even-numbered transmembrane α-helices is usually conserved within MCF, but in OGC it is only partially conserved. The cysteine mutations facing the cytoplasm that inactivated OGC are almost exclusively found in this motif (Fig. 3f) (Miniero et al. 2011), suggesting that they might come together and form the proposed cytoplasmic gate that in carriers with totally conserved motifs would consist of salt bridge network (Robinson et al. 2008).

There are a number of conserved prolines and small amino acids like glycines and alanines within the transmembrane helices that inactivate OGC when replaced by cysteine (Fig. 3g). Many of these residues are found in symmetric and conserved positions in three distinct layers of the carrier. Most probably the prolines of the mitochondrial signature motif sequence and the glycines at the beginning of the even-numbered helices below the substrate binding site as well as the glycines in the [ED]G sequence of the matrix α-helices take part in the opening and closing of the matrix gate. Some of the glycines and alanines above the binding site could mediate close helix-helix contacts created by the conformational changes during the transport cycle (Robinson et al. 2008). For some of the glycines and prolines at specific positions in the odd and even transmembrane α-helices, respectively, above the binding site, it has been proposed that they take part in the transport mechanism by kinking the helices, facilitating opening and closing of the cytosolic gate (Palmieri and Pierri 2010a).

The majority of cysteine replacement mutants that are inhibited by impermeable sulfhydryl reagents, such as methanethiosulfonates (MTS), are found at and around the cytosolic and matrix gates (Fig. 4). In these positions the residues are accessible to the cysteine reactive reagents, at least in certain conformations of the carrier during the transport cycle. Modifications of these residues most likely block the closing or opening of the gates and therefore also substrate transport.

Analysis of the OGC structural model in combination with the cysteine-scanning mutagenesis data fail to identify a potential dimerization interface on the membrane-exposed surface of the carrier, which is functionally important (Fig. 3a–b) (Miniero et al. 2011). In addition, none of the few functionally important residues that are positioned towards the lipid bilayer coincide with conserved and asymmetric residues likely to constitute an evolutionary maintained and specific interface for dimerization.

The systematic cysteine-scanning mutagenesis data of OGC combined with residue conservation analysis of the MCF members and with the OGC structural homology model have revealed sequence-structure-function relationships forming the basis of the recently proposed general transport mechanisms (Palmieri 2012; Palmieri and Pierri 2010a, b; Palmieri et al. 2011; Robinson et al. 2008). The hypotheses suggest that MCs operate as monomers according a centred gated pore mechanism (Klingenberg 1976, 2008), which includes a single centrally located binding site between the cytoplasmic and matrix gates. The substrate enters the cavity, for example, from the cytosolic side, and is selected in the binding site by specific residues that are conserved within each MC subfamily. The binding triggers conformational changes that cause the cytoplasmic gate to close and the matrix gate to open and the substrate is released on the opposite side from where it entered. The opening and closing of the gates is simultaneous so that the carrier is always open towards one side or an occluded state exists in which both gates are closed with the substrate inaccessible from either side. The translocation mechanism is believed to be essentially the same for the entire MCF because many of residues that take part in the interactions for closing and opening the gates are conserved.

Conclusions/future perspective

The well-characterized OGC and the complete cysteine-scanning mutagenesis has made this carrier a model for recognizing which residues are most likely to be of functional importance and involved in substrate binding in all the other members of the MCF. Thereby, these studies have also contributed to the experimental basis for the prediction of substrates for orphan carriers. Furthermore, the suggestion that some crucial residues play essential roles in specific interactions within the structure and the conformational changes occurring during the transport cycle constitute the first pieces of information to understand the puzzle of substrate translocation and molecular events underlying the MC transport mechanism. Future studies of OGC and other carriers will contribute to improve these hypotheses.

Acknowledgements We would like to thank Prof. Ferdinando Palmieri for reading the manuscript critically. This work was supported by grants from MIUR, CNR, CEGBA and the Italian Human ProteomeNet.

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