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Functional and structural role of amino acid residues in the matrix α-helices, termini and cytosolic loops of the bovine mitochondrial oxoglutarate carrier

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ABSTRACT

The mitochondrial oxoglutarate carrier belongs to the mitochondrial carrier family and exchanges oxoglutarate for malate and other dicarboxylates across the mitochondrial inner membrane. Here, single-cysteine mutant carriers were engineered for every residue in the amino- and carboxy-terminus, cytoplasmic loops, and matrix α-helices and their transport activity was measured in the presence and absence of sulfhydryl reagents. The analysis of the cytoplasmic side of the oxoglutarate carrier showed that the conserved and symmetric residues of the mitochondrial carrier motif [DE]XX[RK] localized at the C-terminal end of the even-numbered transmembrane α-helices are important for the function of the carrier, but the non-conserved cytoplasmic loops and termini are not. On the mitochondrial matrix side of the carrier most residues of the three matrix α-helices that are in the interface with the transmembrane α-helical bundle are important for function. Among these are the residues of the symmetric [ED]G motif present at the C-terminus of the matrix α-helices; the tyrosines of the symmetric YK motif at the N-terminus of the matrix α-helices; and the hydrophobic residues M147, I171 and I247. The functional role of these residues was assessed in the structural context of the homology model of OGC. Furthermore, in this study no evidence was found for the presence of a specific homo-dimerisation interface on the surface of the carrier consisting of conserved, asymmetric and transport-critical residues.

1. Introduction

The mitochondrial oxoglutarate carrier (OGC) in the mitochondrial inner membrane plays a central role in the malate/aspartate shuttle, which transfers reducing equivalents from cytosolic NADH to matrix NAD^+ via a series of enzymatic and transport steps. The carrier also participates in the oxoglutarate/isocitrate shuttle, in nitrogen metabolism, and in gluconeogenesis from lactate when the carbon skeleton is provided by oxoglutarate exported from the mitochondrion [1,2]. The function of OGC has been investigated in mitochondria and in liposomes reconstituted with protein purified from native membranes [3–8]. The carrier catalyzes the transport of oxoglutarate in electroneutral exchange for malate or other dicarboxylates, is inhibited by organic mercurials and impermeable substrate analogues, such as phtalonate and phenylsuccinate, and behaves kinetically according to a sequential mechanism.

The determination of the amino acid sequence of OGC [9] showed that this transporter belongs to a large family of related transport proteins, called the mitochondrial carrier family [10–14]. The primary structure of the family members consists of three tandemly repeated homologous domains [15] of about 100 amino acids in length, each containing two hydrophobic segments. Furthermore, a characteristic sequence motif P-X-[DE]-X-X-[RK] is present in all members of the family and in all three repeats (PROSITE PS50920 and PFAM PF00153) [14,16]. The atomic model of the bovine mitochondrial ADP/ATP carrier, which is a member of the protein family, has clarified the structure of these sequence features [17]. Basically, the ADP/ATP carrier 3D structure is composed of six transmembrane α-helices (H1–H6) and three short α-helices (h12, h34 and h56) situated on the matrix side and parallel to the membrane plane. H1–H6 line a water-accessible cavity which is open towards the cytosol and closed on the

Abbreviations: MTSES, sodium(2-sulfonatoethyl)-methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; OGC, oxoglutarate carrier; C-less OGC, cysteine-less OGC; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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matrix side by a salt-bridge network formed by the charged residues of the three signature motifs PX[DE]XX[RK]. This structure corresponds to the cytoplasmic-state (c-state) of the mitochondrial carrier proteins and is three-fold pseudo-symmetric. During their catalytic transport cycle, mitochondrial carriers undergo a reversible transition between the c-state and the matrix state (m-state), in which the internal carrier cavity is open towards the matrix and closed on the cytosolic side by a recently proposed salt-bridge network formed by the charged residues of the three-fold repeated [DE]XX[RK] motif [18].

The OGC has been the first eukaryotic membrane protein to be expressed in *Escherichia coli* and refolded in the reconstitutively active state [19]. This methodology paved the way to using site-directed mutagenesis to elucidate structure-function relationships of this important transporter. These studies showed that the three cysteine residues present in the primary structure of the bovine OGC are not involved in transport mechanism, because the cysteine-less mutant is fully active [8]. More recently, in the cysteine-less mutant of OGC, the residues of the even- and odd-numbered transmembrane α-helices

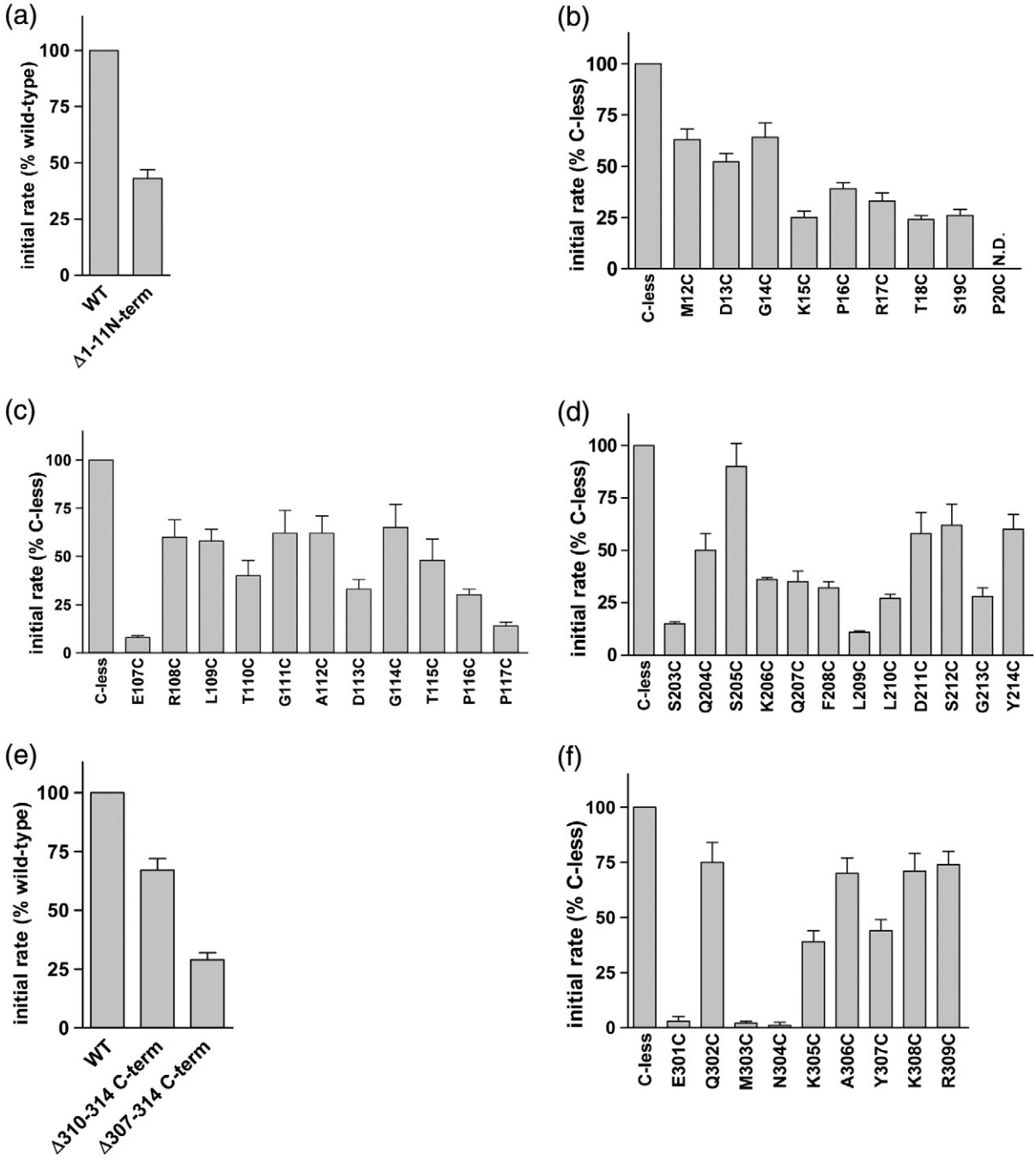

Fig. 1. Initial uptake rate of 2-oxoglutarate by cysteine-less OGC, deletion and single cysteine replacement mutant carriers of the termini, C-terminal ends of H2, H4 and H6 and cytoplasmic loops: (a) deletion of residues 1-11; (b) single Cys replacements of M12 to P20; (c) single Cys replacements of E107 to P117; (d) single Cys replacements of S203 to Y214; (e) deletions of residues 310-314 and 307-314; and (f) single Cys replacements of E301 to R309 (see Fig. S1 in Supplementary material). Transport was started by adding 3 mM 2-oxo-[1-^{14}C]-glutarate to proteoliposomes containing 20 mM 2-oxoglutarate. Initial uptake rates of oxoglutarate/oxoglutarate exchange were determined after 30 s of uptake. The data are expressed as percentage of the cysteine-less OGC value, which was on average 2219 ± 356 nmol min^{-1} mg^{-1} of protein. Data relative to mutants from S203 to S205 and from E301C to K305C were taken from refs. [8,21]. The transport activity of mutant P20C, which was not expressed, could not be assayed (N.D., not determined). The means ± S.D. of at least three independent experiments carried out in duplicate are shown.

have been replaced with cysteine and other amino acids [20,21]. In conjunction with labeling studies of single Cys mutants with the highly hydrophilic methanethiosulfonate SH-blocking reagents, this systematic study has led to the identification of residues that are important for the function of the carrier [20,21]. In particular, residues that are in the area of the proposed substrate binding site [22,23] and in the signature motifs of the mitochondrial carrier family members were found to be critical for function [20,21].

Here, the role of residues in the termini, cytoplasmic loops and matrix α-helices of OGC was investigated, completing the cysteine-scanning analysis of this important carrier. The present study highlights several amino acid positions located on the matrix and cytosolic sides of OGC that are critical for the function of this carrier and relevant for the transport mechanism. Furthermore, the study shows that none of the residues on the surface of OGC are likely to be competent to form a specific homo-dimerisation interface.

2. Materials and methods

2.1. Materials

2-Oxo[1-^{14}C]glutaric acid was purchased from Dupont De Nemours (Milan, Italy); PIPES, Triton X-114, Amberlite XAD-4 and egg yolk phospholipids (lecithin from eggs) from Fluka (Milan, Italy); N-

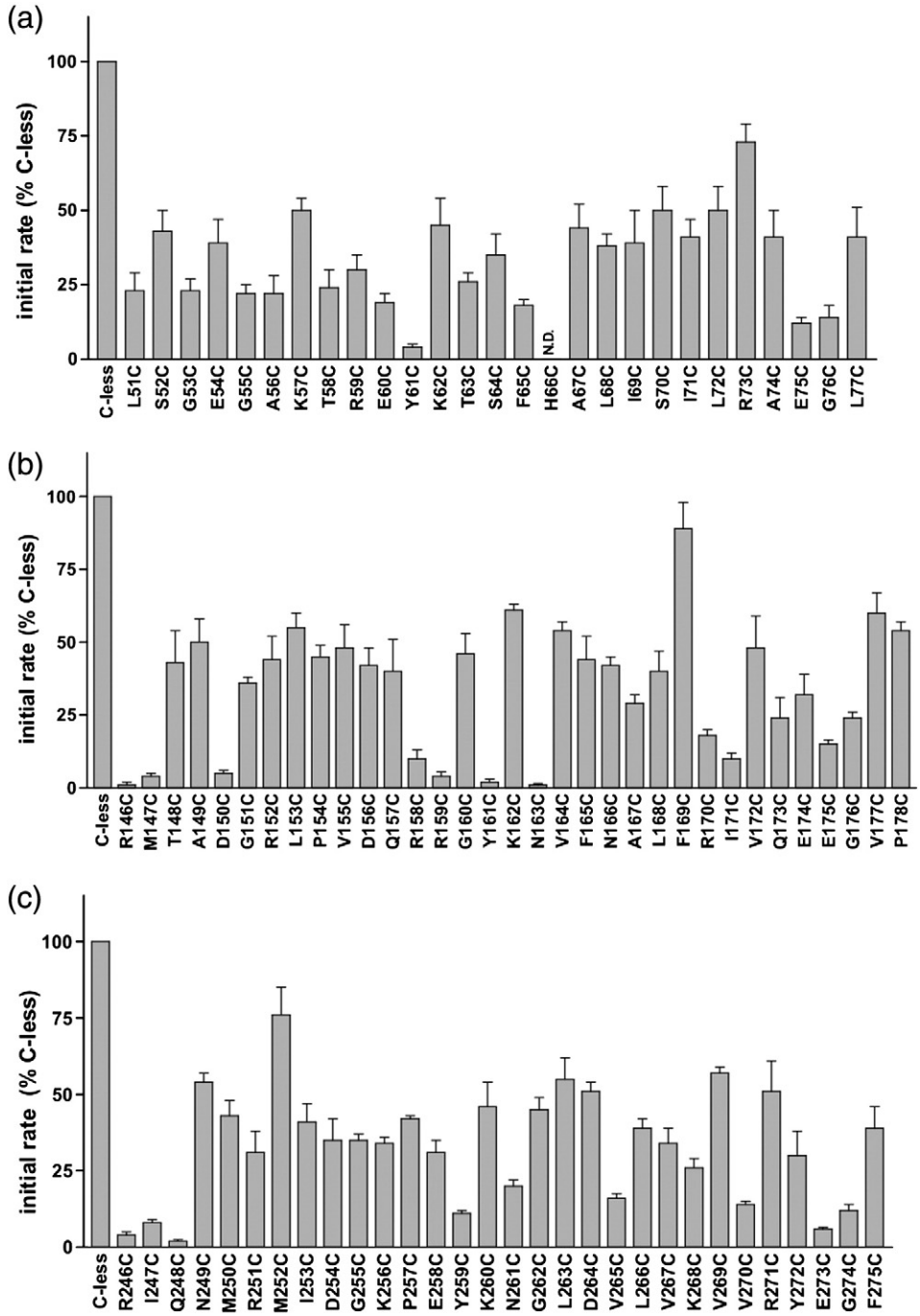

Fig. 2. Initial uptake rate of 2-oxoglutarate by cysteine-less OGC and by single cysteine-replacement mutant carriers for matrix α-helices h12, h34 and h56. The single-letter amino acid code along the horizontal axis denotes the original residues replaced with Cys from L51 to L77 in matrix α-helix h12 (a), from R146 to P178 in matrix α-helix h34 (b), and from R246 to F275 in matrix α-helix h56 (c). The experimental details are described in the legend of Fig. 1. The transport activity of mutant H66C, which was not expressed, could not be assayed (N.D., not determined). The means ± S.D. of at least three independent experiments carried out in duplicate are shown.

dodecanoylsarcosine (sarkosyl) from Sigma (Milan, Italy); MTSES and MTSET from Fluorescent Dyes Inc. (Toronto, Canada); and Sephadex G-75 from Pharmacia (Milan, Italy). All other reagents were of analytical grade.

2.2. Mutagenesis and functional analysis of OGC

The cloning, expression, purification and reconstitution of recombinant OGC from sarkosyl were carried out as described previously [20,21]. To assay the reconstitution efficiency, the amount of OGC incorporated into liposomes was estimated as described [24]. The functional analysis in the presence and absence of sodium-(2-sulfonatoethyl)-methane-thiosulfonate (MTSES) or [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) was carried out as described [20,21].

2.3. Analysis of amino acid substitutions and structural mapping

The OGC orthologs were identified in the following organisms: *Anopheles gambiae*, *Drosophila melanogaster*, *D. pseudoobscura*, *Caenorhabditis elegans*, *C. briggsae*, *Bos taurus*, *Homo sapiens*, *Oryza sativa*, *Pan troglodytes*, *Pongo pygmaeus*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Xenopus laevis* and *Tetraodon nigroviridis*. The sequences were obtained from UniProt (http://www.ebi.uniprot.org/). The amino acid sequences of the orthologs were aligned with CLUSTALW [25] and the number of amino acid substitutions per position was counted. The amino acid substitution and the effects of the mutations were mapped on a structural model of the bovine OGC, which was built based on the structure of the bovine AAC1, as described [21].

3. Results

3.1. Transport activity of reconstituted single cysteine OGC mutants

Each amino acid residue on the cytoplasmic and matrix side of OGC was replaced with a cysteine by using a functional cysteine-less OGC as a template. The residues which have been substituted are indicated in black in the OGC topology model (see Fig. S1 in Supplementary material). The expression levels of the mutant OGC, overproduced in *Escherichia coli*, ranged between 52% and 98% as compared to those of the wild-type OGC, except for H66C and P20C, which were not expressed at all. The

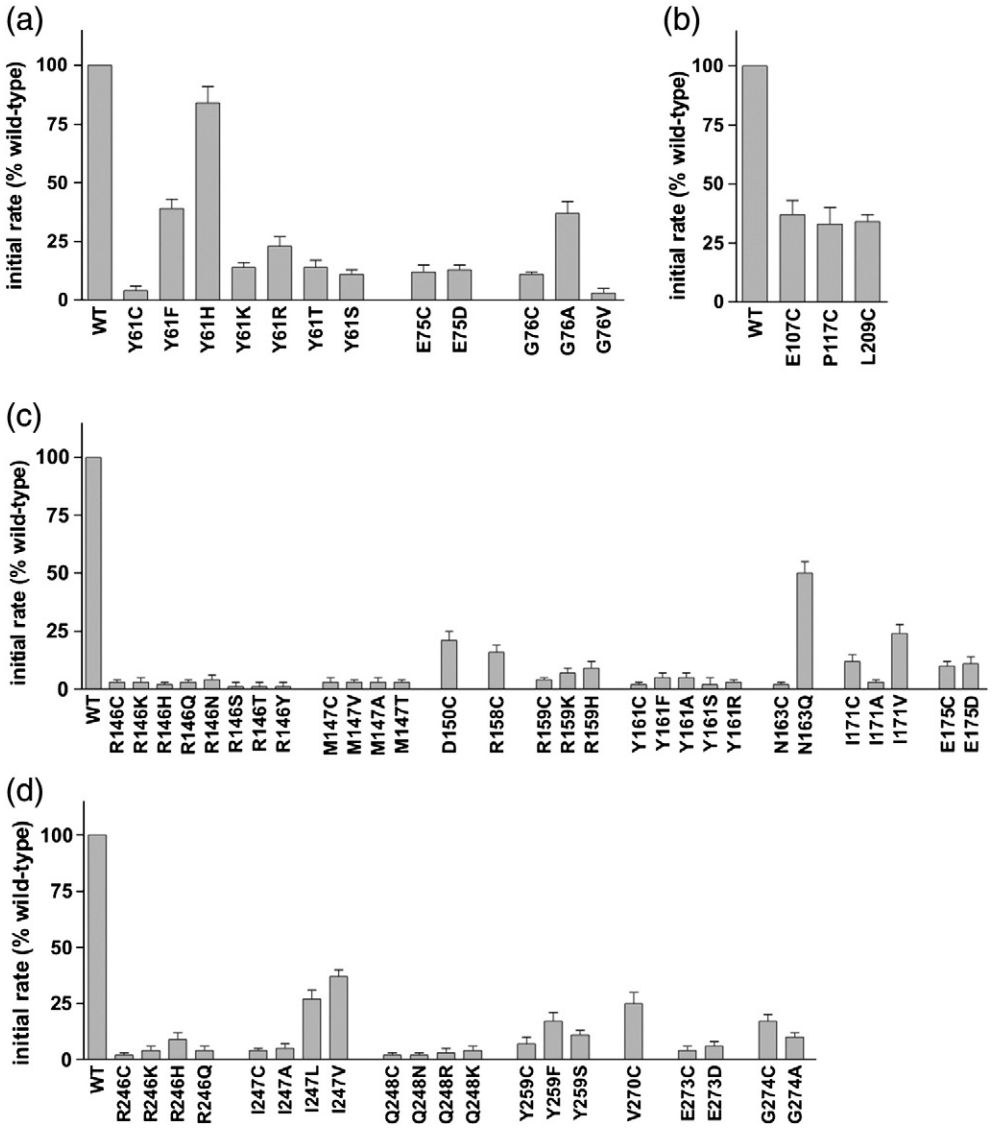

Fig. 3. Transport of 2-oxoglutarate by single replacement mutants in the wild-type OGC background. The single-letter amino acid code along the horizontal axis denotes the specific residue replacements in matrix α-helix h12 (a), C-terminal end of H2 and cytosolic loops between H2-H3 and H4-H5 (b), matrix α-helix h34 (c) and matrix α-helix h56 (d). Transport was started by adding 3 mM 2-oxo-[1-^{14}C]-glutarate to proteoliposomes containing 20 mM 2-oxoglutarate. Initial uptake rates of oxoglutarate/oxoglutarate exchange were determined after 30 s of uptake. The data are expressed as percentage of the wild-type OGC value, which was 2723 ± 392 nmol min^{-1} mg^{-1} of protein. The means ± S.D. of at least three independent experiments carried out in duplicate are shown.

recombinant proteins were refolded and reconstituted into liposomes and the yield of reconstituted wild-type and mutant proteins varied between 18% and 35% of the added protein. The initial transport rates of 2-oxoglutarate for each recombinant OGC were measured and expressed as a percentage of the value for the cysteine-less carrier for the cytoplasmic (Fig. 1) and matrix side (Fig. 2).

The first 11 residues of OGC were not critical for the function of the carrier, as they could be deleted collectively without abolishing activity (Fig. 1a). The single cysteine replacements of the following eight residues of the N-terminus reduced incrementally the transport activity, but the transporter remained functional (Fig. 1b). These results show that the N-terminus was not critical for function of the carrier. Very few cysteine replacements in the C-terminal ends of the even-numbered α-helices and in the cytoplasmic loops affected transport activity (Figs. 1c and d). Among the ones with a severe effect on transport were E107C, P117C, S203C and L209C in the C-terminal ends of α-helices H2 and H4. The C-terminus was also not critical for function as residues 310–314 or 307–314 could be deleted from OGC without severe empairment of activity, i.e., transport rate higher than 15% as compared to that of the wild-type carrier (Fig. 1e). Also, the individual cysteine replacements in this region did not lead to an inactivated carrier from residues R309 to K305 (Fig. 1f). On the contrary, single cysteine replacements of the preceding residues E301, M303 and N304 resulted in inactive carriers (Fig. 1f). On the matrix side of OGC the vast majority of cysteine replacements that affected the function of the carrier were in the interface between the matrix α-helices and odd-numbered α-helices (Fig. S2 in Supplementary material), including those of residues Y61, E75, and G76 on matrix α-helix h12 (Fig. 2a), R146, M147, D150, R158, R159, Y161, N163, I171 and E175 on matrix α-helix h34 (Fig. 2b), and R246, I247, Q248, Y259, V270, E273, G274 on matrix α-helix h56 (Fig. 2c). The effects of the mutations on the initial transport rate were mapped on the comparative model of OGC (Fig. S2 in Supplementary material).

To exclude the possibility that the loss of transport activity was due to combinations of mutations many of the above-mentioned mutations were cloned in a wild-type gene background also (Fig. 3). Most single cysteine replacements in wild-type OGC had a similar effect on transport as in the cysteine-less OGC, except for E107C, P117C, D150C, L209C (Figs. 3b and c), V270C (Fig. 3d), M303C and N304C [21] where the effect was less severe, i.e., between 20% and 40% of the initial rate of the wild-type. On the whole, conservative replacements, such as Y61F, G76A, N163Q, I171V and I247L, had smaller effects on transport than cysteine replacements. The Y61H mutation did not affect transport much either, as Tyr and His are both polar and aromatic residues.

3.2. Effect of sulfhydryl reagents on the transport activity of OGC mutants

The negatively charged MTSES and positively charged MTSET sulfhydryl reagents, which are both membrane impermeable [26,27], were used to probe the effect of cysteine modifications on transport. The residual transport activity after modification with MTSES and MTSET is shown in Figs. 4 and 5 for all but P20C and H66C, which were not expressed, and the mutant carriers that lacked transport activity. The mutants S205C, Y214C, L51C, L68C, I71C, T148C, A149C, N249C,

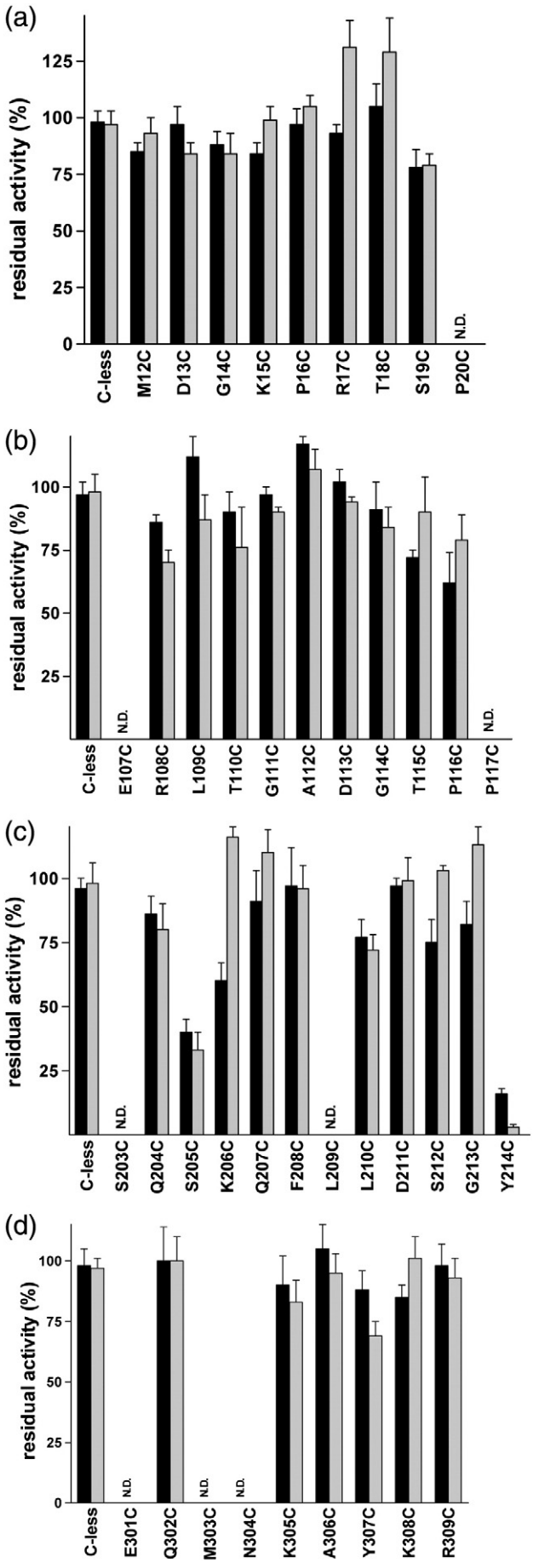

Fig. 4. Effect of sulfhydryl reagents on the rate of 2-oxoglutarate uptake by cysteine-less OGC or by single cysteine-replacement mutant carriers on the cytoplasmic side. Shown along the horizontal axis are the cysteine replacements in the N-terminus (a), C-terminal end of α-helix H2 and cytoplasmic loop between H2 and H3 (b), C-terminal end of α-helix H4 and cytoplasmic loop between H4 and H5 (c), and C-terminal end of α-helix H6 and C-terminus (d). Proteoliposomes were preincubated in the presence or absence of 5 mM MTSES or MTSET and transport was started by adding 0.3 mM 2-oxo-[1-^{14}C]-glutarate to proteoliposomes containing 20 mM 2-oxoglutarate. Initial uptake rates of oxoglutarate/oxoglutarate exchange were determined after 30 s of uptake. The black and grey bars represent the percent of the activity in the presence of MTSES or MTSET, respectively, with respect to the control value without inhibitor. Mutant carriers that were severely affected by the cysteine replacement and P20C, which was not expressed, could not be assayed (N.D., not determined).

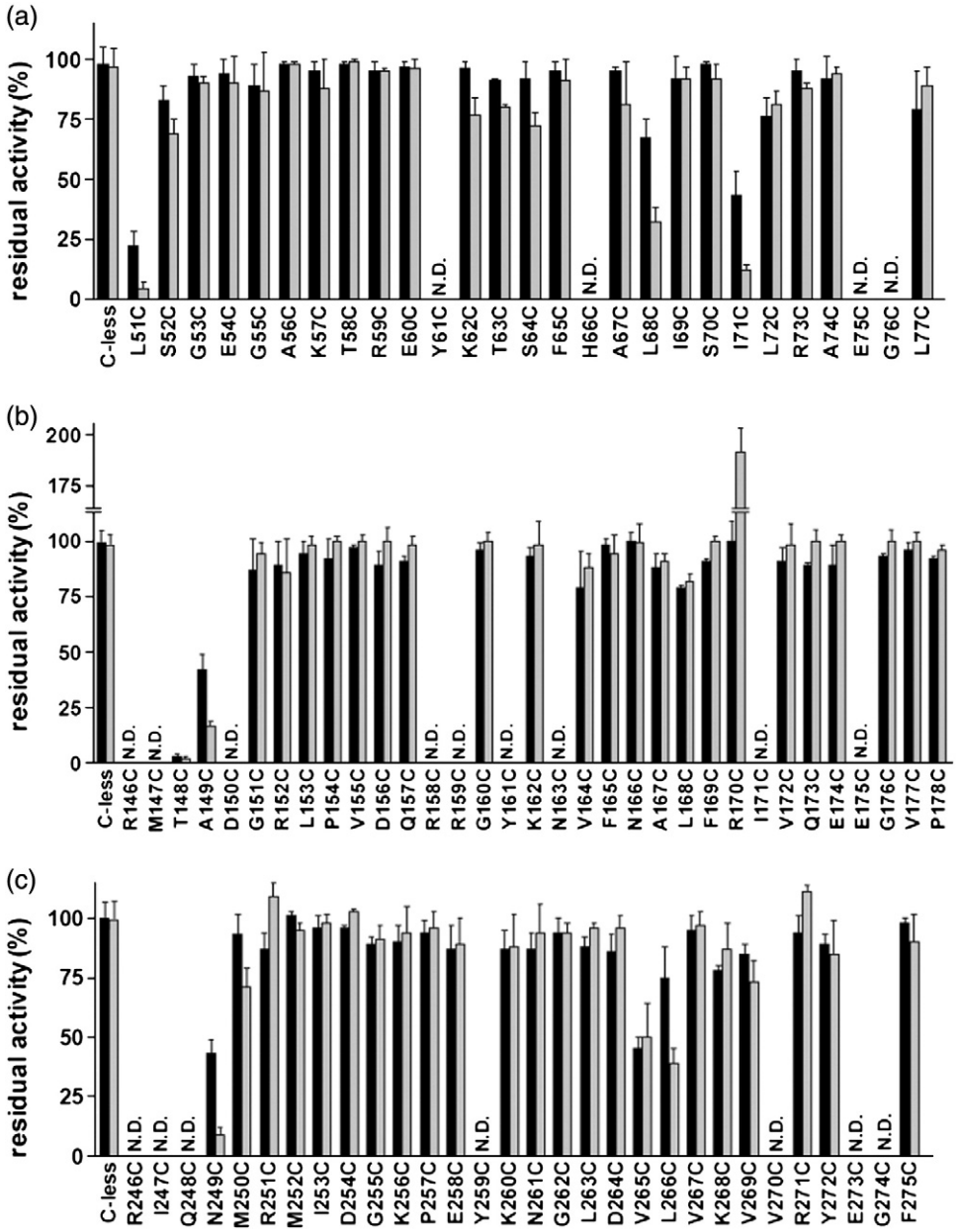

Fig. 5. Effect of sulfhydryl reagents on the rate of 2-oxoglutarate uptake by cysteine-less OGC or by single cysteine-replacement mutant carriers of the matrix α-helices h12, h34, and h56. Shown along the horizontal axis are the cysteine replacements in matrix α-helix h12 (a), h34 (b), and h56 (c). The experimental details are described in the legend of Fig. 4. Mutant carriers that were severely affected by the cysteine replacement and H66C, which was not expressed, could not be assayed (N.D., not determined).

V265C and L266C were significantly inactivated by modification with MTSES and/or MTSET. Remarkably, the modification of R170C with the positively charged MTSET stimulated the transport rate of the mutant carrier two-fold (Fig. 5). However, sulfhydryl modification of residues S70 and K268, which are in equivalent positions of R170C in the other repeats, did not affect carrier activity (Fig. 5). The results obtained with MTSES were mapped on the structural model of OGC (Fig. S3 in Supplementary material).

3.3. Conservation of OGC orthologs

In general, the N- and C-termini of mitochondrial carriers are variable in length and composition and the subfamily of oxoglutarate carriers is no exception (Fig. S4 in Supplementary material). The two cytoplasmic loops, following H2 and H4, respectively, are also not strictly conserved between OGC orthologs (Fig. S4 in Supplementary material). However, the matrix α-helices have a relatively well-conserved face, which forms the interface with the transmembrane α-helices, and a face with low conservation, which interacts with the membrane and the water phase of the mitochondrial matrix (Fig. S4 in Supplementary material).

4. Discussion

In this study, the cysteine-scanning analysis of OGC has been completed, and the functional relevance of the currently investigated carrier regions has been assessed in the structural context of the OGC homology model [20,21] based on the bovine ADP/ATP carrier [17].

4.1. C-terminal end of the odd-numbered α-helices of the oxoglutarate carrier

In previous work [20], we observed that Cys-substitution of Q50 resulted in inactivation of the OGC. The results presented here show that the symmetry-related residues Q248 and T148 are also inactived by Cys

replacement (Fig. 2) and sulphydryl modification (Fig. 5), respectively. These three residues are likely to be interacting with the charge residues of the matrix salt-bridge network [16,17], which is involved in the opening/closing of the mitochondrial carrier gate on the matrix side [11,18]. In addition, Cys replacement of the neighbouring residues L51, A149, and N249 does not affect the function of OGC (Fig. 2), but the subsequent modification of the cysteines with MTSES and MTSET at these positions markedly reduces the OGC transport activity (Fig. 5). Residues L51, A149, and N249 are most likely in the passage way for the substrate (Fig. 6d). Thus, they become exposed when the carrier opens towards the matrix side and labeling of these mutant proteins with methanethiosulphonate SH-blocking reagents lead to inhibition of transport.

4.2. The matrix α-helices of the mitochondrial oxoglutarate carrier

The matrix α-helices link the odd- and even-numbered transmembrane α-helices in each repeat [17], but their function is not yet understood. Most residues of the OGC matrix α-helices are not critical for function (Fig. 2) in agreement with the conservation (Fig. S4 in Supplementary material) and symmetry analysis [18]. Residues important for function are found in the interface of the matrix α-helices with the odd-numbered α-helices (Fig. 6e). Among them are the residues Y61, E75, G76, Y161, E175, G176, Y259, E273 and G274 of the YK and [ED]G motifs that upon substitution with cysteine cause a significant impairment of the carrier's transport ability (Fig. 2). The inhibition of OGC activity by Cys replacement of E at position 75, 175 and 273 are the first evidence in favour of a putative salt bridge in OGC between the glutamates and the conserved and three-fold repeated positively charged residue, two residues C-terminal of the signature motif PX[ED]XX[RK], as found in the crystalline structure of the ADP/ATP carrier [17]. The hydroxyl groups of tyrosines Y61, Y161 and Y259 are in the vicinity of the matrix salt bridge and might interact with these residues (Fig. 6e). G76, G176 and G274 are highly conserved in the whole mitochondrial carrier family and may provide a flexible link between the matrix and the short linker α-helices, which are present at the N-terminal end of the even-numbered α-helices (Fig. 6e). The role of residues M147, I171 and I247 at the interface between the matrix and transmembrane α-helices is not clear, but their position might indicate that mutations of these residues could disrupt this interface (Figs. 2 and 6e). In addition, sulfhydryl modifications of I71C and V265C partially prevent the carrier from functioning (Fig. 5), indicating that the accessibility of the interface might change during the transport cycle, as was also proposed for the ADP/ATP carrier [28].

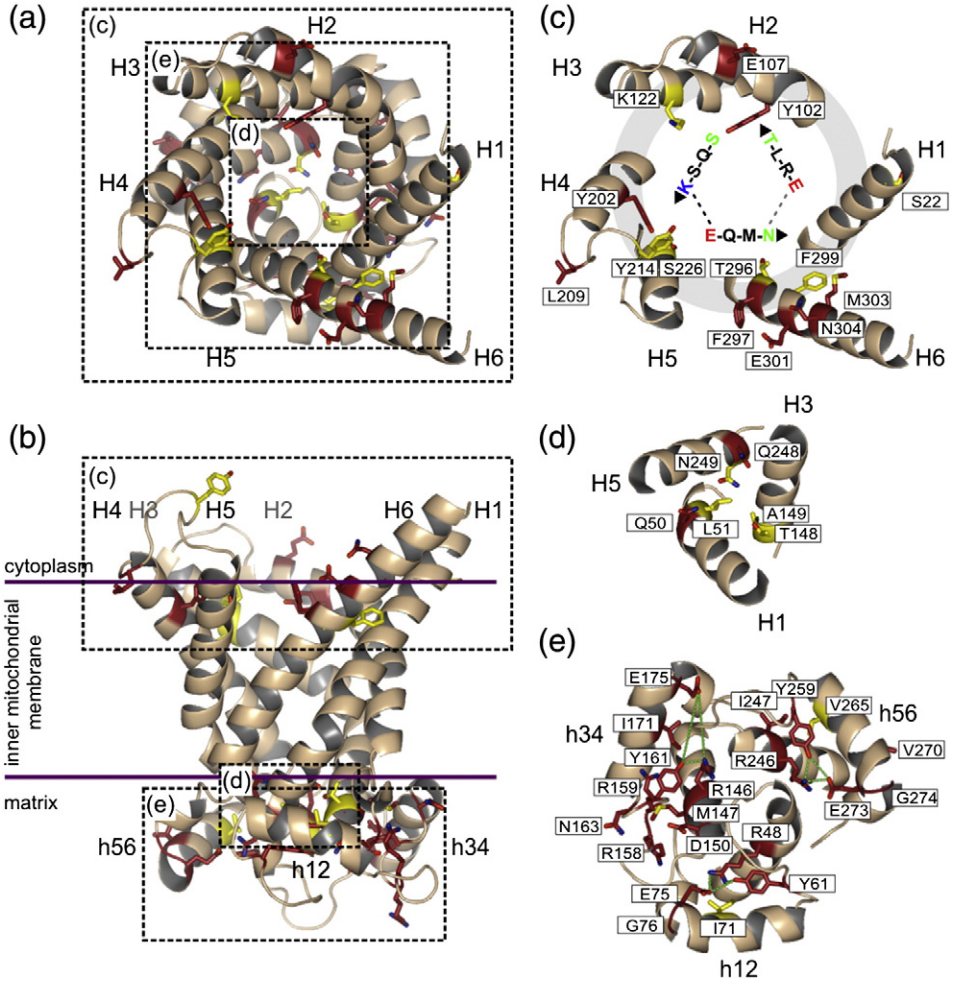

Fig. 6. Overview and details of the areas that most affect the function of OGC after cysteine replacement or modification with sulfhydryl reagents. (a) Cytoplasmic and (b) lateral view from the membrane of the comparative model of OGC based on the structure of the mitochondrial ADP/ATP carrier. Cysteine replacements that abolish transport activity are coloured red, whereas cysteine replacements that abolish transport activity after labeling with sulfhydryl reagents are coloured yellow. Dashed boxes indicate the three sections shown in panel (c), (d) and (e). Panels (c) and (e) show the cytoplasmic side and matrix side of the structural model of OGC, respectively, whereas panel (d) shows the area just beneath the matrix salt bridge network. The residues are labelled also with their one-letter amino acid code and their residue number. Panel (c) shows the putative interactions of the cytoplasmic network in the matrix state schematically. In panel (e) putative interactions between residues are shown in green dotted lines. The results for residues R48 and Q50 have been reported previously [20], but they have been added because they are pseudo-symmetrically related to residues R146 and R246, and T148 and Q248, respectively.

4.3. The cytoplasmic side of the oxoglutarate carrier

On the cytoplasmic side of mitochondrial carriers the residues of the motif [DE]XX[RK] localized at the C-termini of the even-numbered α-helices may form a salt-bridge network as part of the cytosolic gate in the matrix state [11,18]. In the bovine OGC the residues of this motif are partially conserved: ERLT in H2, SQSK in H4, and EQMN in H6 (Fig. 6c). The residues T110 and S203 as well as N304 and E107 might form hydrogen bonds instead, although it is not clear whether the distance between the side chains would be sufficient (Fig. 6c). It is notable that replacement with cysteine of E107, S203, E301 and N304 causes a severe inactivation of OGC (Fig. 1), although in the case of E107C and N304C the inhibition is less pronounced in the wild-type than in the cysteine-less background. It is worth mentioning that modification of the cysteines replacing residues K122 [20], Y214 (Fig. 4), S226 [20], T296 and F299 [21] with sulfhydryl reagents lead to a dysfunctional carrier. Given that all of these residues are very close to the hypothesized cytosolic gate, it is possible that these modifications interfere with the closure/opening of OGC on the cytosolic side in the transport cycle. It should be added that we have previously reported that the mutants Y102C, Y202C and F297C exhibit an almost complete inhibition of oxoglutarate transport [8,21]. These aromatic residues precede the [DE]XX[RK] motif, discussed above, and are highly conserved and three-fold symmetric in members of the mitochondrial carrier family. They could be involved in the proposed cytosolic gate, as they are bulky aromatic residues that could close the carrier in the matrix state.

4.4. Search for a dimerisation interface

The oxoglutarate carrier, like other mitochondrial carriers, was believed to exist and function as a homo-dimer [29,30]. The inner membrane of mammalian mitochondria contains around 50 different mitochondrial carriers [10–14,31]. If specific homo-dimers were to form in the presence of many structurally related but functionally different carriers, one would expect that dimerisation be mediated by specific protein-protein interactions. The completion of the cys-scanning mutagenesis of OGC permits analysis of putative dimerisation interfaces since their residues are likely to be critical for transport, conserved within the OGC subfamily, asymmetrically distributed (since three-fold symmetrical residues are incompatible interfaces for a dimer) and clustered together on the surface. There are only 26 functionally important residues on the surface of OGC (Fig. 7a), but most belong to the matrix gate, the recently proposed cytoplasmic gate, the EG motif at the C-terminal end of the matrix α-helices, and the [WYF][RK]G motif at the N-terminus of the even-numbered α-helices. The remaining

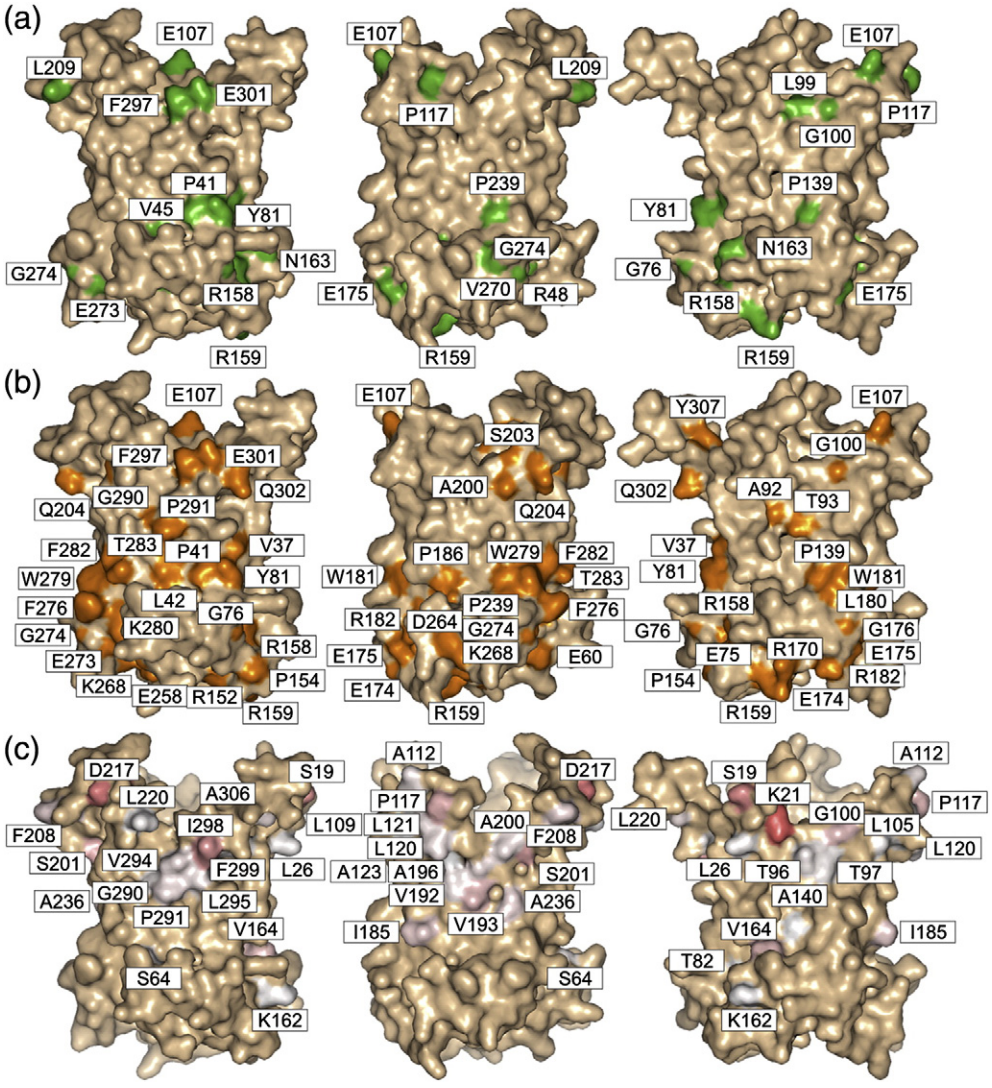

Fig. 7. Conserved, asymmetrical and transport-critical residues on the surface of the bovine oxoglutarate carrier. Surface representation of the comparative model of OGC showing (a) residues critical for function in green, (b) conserved residues in orange, and (c) asymmetric residues in a colour scale from white (neutral) to red (highly asymmetric) [18]. The views are related to each other by 120° rotation to emphasise the three-fold pseudo-symmetry.

residues are: L99 and G100 [21], P117 and L209 (Fig. 1), and V270 (Fig. 2) that do not have the ability to form strong and specific interactions; R158, R159 (Fig. 2) (in the loop region between transmembrane α-helix H3 and matrix α-helix h34) that are both positively charged; and N163 (Fig. 2) which is not conserved. Thus, none of the functionally relevant residues located on the surface of OGC are likely to be involved in a specific homo-dimer interaction. Consistent with this conclusion, the conservation analysis shows that most of the 57 strictly conserved amino acid residues on the surface of OGC (Fig. 7b) are found in the above-mentioned motifs that are present in all mitochondrial carriers and in all sequence repeats and thus cannot form a specific interaction interface for homo-dimerisation of OGC. The remaining conserved residues are glycines and prolines, such as G100, P154, P186, G290 and P291 which form kinks [11] and inter-helical interactions, and hydrophobic (V37, A92, A200, L180, F276, and F282) and polar residues (T93, Q204, T283 and Q302) that are widespread on the OGC surface and thus unlikely to form a specific interface. Finally, a third criterion used to inspect the residues on the surface of OGC is the deviation from three-fold pseudo-symmetry, which is required for specific homo-dimerisation. The asymmetric residues of the loop regions on the cytoplasmic and matrix side are not conserved in the OGC subfamily, and thus unlikely to be involved in dimerisation. Therefore, S19, K21, G100, P117, V164, I185, V193, S201, D217 and I298 are the only asymmetric residues that might be potential candidates for specific recognition (Fig. 7c). Most strikingly, none of these asymmetric residues, apart from G100, is conserved and critical for function. On the basis of all these criteria, there is no evidence for the presence of a homo-dimerisation interface in OGC.

4.5. Concluding remarks

In conclusion, the systematic mutagenesis of the mitochondrial oxoglutarate carrier has now been completed, allowing for the first time the identification of all of the areas that are critical for its function. The most critical residues can be found in the matrix salt-bridge network and in its immediate environment. These residues are involved in the formation of the matrix gate, but also in the interactions with the matrix α-helices, showing that they play a central role in the transport mechanism. Another set of critical residues belong to the recently hypothesized cytoplasmic salt-bridge network, which could form the cytoplasmic gate when the substrate binding site is accessible from the mitochondrial matrix. Finally, in the central part of the cavity in between the two networks are the residues of the substrate binding site, which cannot be changed or modified either. The function and structural arrangement of the matrix gate is consistent with the crystal structure of the ADP/ATP carrier, but the hypothesized cytosolic gate and substrate binding site need to be confirmed by structural data.

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