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The substrate specificity of mitochondrial carriers: Mutagenesis revisited

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Abstract

Mitochondrial carriers transport inorganic ions, nucleotides, amino acids, keto acids and cofactors across the mitochondrial inner membrane. Structurally they consist of three domains, each containing two transmembrane α-helices linked by a short α-helix and loop. The substrate binds to three major contact points in the central cavity. The class of substrate (e.g., adenine nucleotides) is determined by contact point II on transmembrane α-helix H4 and the type of substrate within the class (e.g., ADP, coenzyme A) by contact point I in H2, whereas contact point III on H6 is most usually a positively charged residue, irrespective of the type or class. Two salt bridge networks, consisting of conserved and symmetric residues, are located on the matrix and cytoplasmic side of the cavity. These residues are part of the gates that are involved in opening and closing of the carrier during the transport cycle, exposing the central substrate binding site to either side of the membrane in an alternating way. Here we revisit the plethora of mutagenesis data that have been collected over the last two decades to see if the residues in the proposed binding site and salt bridge networks are indeed important for function. The analysis shows that the major contact points of the substrate binding site are indeed crucial for function and in defining the specificity. The matrix salt bridge network is more critical for function than the cytoplasmic salt bridge network in agreement with its central position, but neither is likely to be involved in substrate recognition directly.

Keywords: *Substrate binding, transport mechanism, mitochondrial carriers, transport proteins, alternating access model*

Introduction

Many transport steps across the mitochondrial inner membrane are required for the generation of metabolic energy from the oxidation of sugars and fats, synthesis of haem and iron sulphur clusters, production of heat, macromolecular synthesis and breakdown, and the synthesis, degradation and interconversion of amino acids. Members of the mitochondrial carrier family (MCF) are involved in the majority of these transport steps (Palmieri 2004, 2012), but the transport of pyruvate is carried out by the MPC family (Herzig et al. 2012). Some MCF members have also been found in membranes of other organelles, such as peroxisomes and chloroplasts (Palmieri et al. 2011). On the protein level, mitochondrial carriers are characterized by having three homologous repeats, consisting of about a hundred amino acid residues each (Saraste and Walker 1982). The structural fold of the bovine ADP/ATP carrier, the only member of the family for which an atomic structure is available, consists of a barrel of six transmembrane α-helices (H1-6) with three short α-helices in the matrix loops (h12, h34, h56), which are arranged in a three-fold pseudo-symmetrical manner (Pebay-Peyroula et al. 2003). At the carboxy-terminal ends of all odd-numbered α-helices H1, H3 and H5 a highly conserved signature motif PX[DE]XX[KR] is found (Nelson et al. 1998, Pebay-Peyroula et al. 2003). The prolines of the signature motifs are present at sharp kinks in the helices, whereas the charged residues form three salt bridges, which close the cavity to the matrix side. The structure provided the basic fold of all mitochondrial carriers but did not reveal the location of the substrate binding site nor the putative movements upon substrate binding, as the

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structure is representing an inhibited state, i.e., with carboxy-atractyloside bound.

Computational approaches to identify the substrate binding site

Mitochondrial carriers have a similar structure but can handle a large variety of substrates ranging from single protons (uncoupling proteins) to large cofactors, such as NAD^+ and coenzyme A (Palmieri 2004, 2012). The substrate binding site of mitochondrial carriers was located by three different computational methods. The first used comparative structural models of carriers with known substrate specificity combined with chemical and distance constraints to identify conserved substrate binding sites that were capable of discriminating between (oxo)carboxylates, amino acids and nucleotides (Kunji and Robinson 2006, Robinson and Kunji 2006). It was discovered that there are three main contact points involved in substrate binding, which were indicated by roman numerals I, II and III. The contact points may involve one or several residues, positioned approximately in the middle of the membrane, on the cavity-exposed face of the even-numbered α-helices. For the bovine mitochondrial ADP/ATP carrier, the contact points were proposed to be R79 on H2 (contact point I) and R279 on H6 (contact point III) for binding of the phosphate groups and G182, I183 and Y186 on H4 for binding of the adenine moiety (contact point II) (Kunji and Robinson 2006, Robinson and Kunji 2006). The interactions with the contact points allow the coupling of substrate binding to a symmetrical mechanism.

The second approach used molecular dynamics simulations to identify residues of the bovine mitochondrial ADP/ATP carrier that are involved in the trajectory and binding of ADP in the cavity. ADP bound with a minimum of the free energy to the binding site defined above, revealing an aromatic stacking arrangement of the adenine moiety with Y186 and an ionic interaction of the β-phosphate of ADP with an arginine, which comes through the centre of the matrix salt bridge network (Dehez et al. 2008, Wang and Tajkhorshid 2008). From a sequence perspective, it was clear that the most important interactions of the carriers with the substrate were electrostatic in nature (Kunji and Robinson 2006), but this notion has been refined by calculating electrostatic potentials (Wang and Tajkhorshid 2008) and by looking at the effects of chlorine ions on binding (Krammer et al. 2009).

The third approach exploited the principle that mitochondrial carriers have a high degree of threefold pseudo-symmetry in contrast to the transported substrates that are asymmetric in structure and chemistry (Robinson et al. 2008). Therefore, the residues involved in substrate binding must deviate from each other to couple the binding of the asymmetric substrate to a symmetric transport mechanism. Conserved asymmetric residues were found to cluster consistently at a single site that overlapped with the common substrate binding site in all studied mitochondrial carriers (Kunji and Robinson 2006, Robinson and Kunji 2006, Robinson et al. 2008). In addition, conserved negatively charged asymmetric residues were observed in the substrate binding sites of carriers that transport substrates into mitochondria by coupling the transport step to the import of protons, indicating a potential link between proton and substrate binding (Kunji and Robinson 2010).

Mutagenesis studies

Many mutagenesis studies on mitochondrial carriers had been carried out prior to any structural information being available. Here we revisit the effects of these mutations to see if they are consistent with the proposed substrate binding site. In these studies the activity of the mutant proteins have been assessed by transport experiments in reconstituted liposomes (Fiermonte et al. 1993, Palmieri et al. 1996a) or by mutant complementation assays in deletion strains of yeast (Nelson et al. 1993). So far, the bovine 2-oxoglutarate carrier OGC is the only mitochondrial carrier in which every residue has been mutated to cysteine and to other amino acids when the Cys-replacement leads to loss of transport (Stipani et al. 2001, Cappello et al. 2006, 2007, Miniero et al. 2011). These systematic studies have carved out the conserved functional elements of mitochondrial carriers. The OGC studies demonstrate that the vast majority of the critical residues have their side chains protruding into the cavity of the carrier, whereas only a few critical residues are found on the surface exposed to the mitochondrial membrane, in the cytoplasmic loops and in the matrix helices (Miniero et al. 2011). Extensive mutagenesis studies in other carriers have been carried out on cavity residues of the yeast phosphate carrier Mir1 (Wohlrab and Briggs 1994, Phelps et al. 1996, Briggs et al. 1999, Wohlrab et al. 2002) and the yeast citrate carrier Ctp1 (Kaplan et al. 2000, Ma et al. 2004, 2007), whereas only selected residues have been investigated in the yeast ADP/ATP carrier Aac2 (Nelson et al. 1993, 1998, Heidkämper et al. 1996, David et al. 2008), the human folate carrier (Lawrence et al. 2011), the human and rat carnitine/acyl-carnitine carrier

(Indiveri et al. 2002, De Lucas et al. 2008, Tonazzi et al. 2009, Giangregorio et al. 2010, Tonazzi et al. 2012), and the human and rat uncoupling protein (Echtay et al. 1997, 2001, Modrianský et al. 1997). Since the latter mutagenesis studies are incomplete, we have chosen to focus on the cavity residues of OGC, Mir1 and Ctp1 to evaluate if the computational approaches agree with the results of mutagenesis studies. Effects of mutations on the function of the ADP/ATP carrier have been described elsewhere (Nury et al. 2006).

Analysis of mutations of residues in the substrate binding site

The pseudo-symmetry and conservation analysis of OGC (Figure 1A and C), Mir1 (Figure 2A and C) and Ctp1 (Figure 3A and C) demonstrate that the

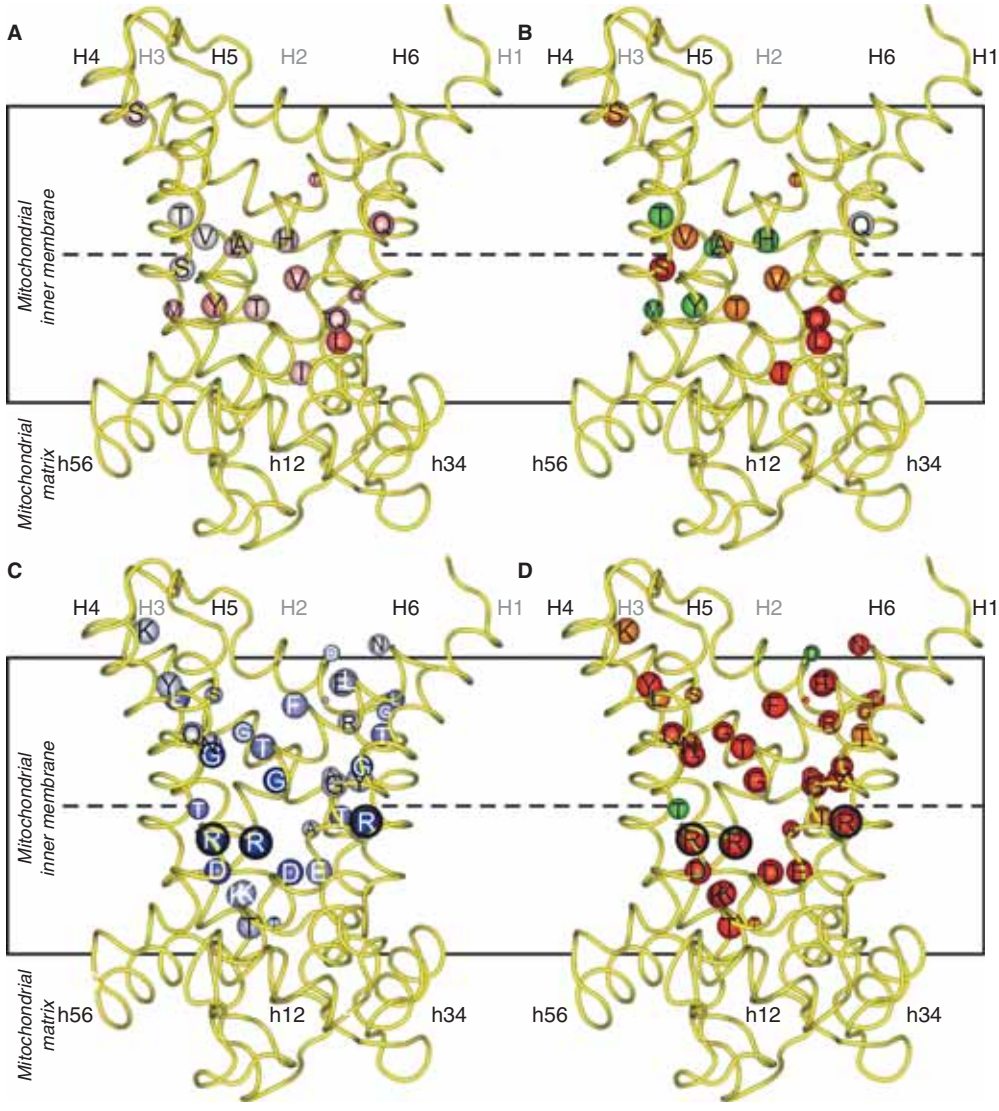

Figure 1. Asymmetry, symmetry and the effects of single cysteine mutations on transport activity of the bovine 2-oxoglutarate carrier OGC. The backbone is shown in yellow and is based on the structure of the bovine AAC1 (Pebay-Peyroula et al. 2003). The conservation and average symmetry scores of the OGC subfamily are represented by the size and colour of the Cβ atom, respectively. Large spheres indicate residues that are well-conserved in the subfamily of OGC, whereas small spheres are not. Asymmetric residues are shown in a colour scale from red (highly asymmetric) to white (neutral) (A), whereas symmetric residues are shown in a colour scale from blue (highly symmetric) to white (neutral) (C) (Cappello et al. 2006). The asymmetric (B) and symmetric residues (D) were also coloured according to the relative initial transport velocity of the single cysteine mutant proteins compared with the wild-type at the external substrate concentration equal to the km of the wild-type: red, 0–15%; orange 16–50%; green, 51–100%; white, no data. The black encircled residues are the three contact points of the substrate binding site, which in OGC is a symmetrical triplet of arginines. As the substrates malate and oxoglutarate are small, the substrate binding site has only a few asymmetrical adaptations. This Figure is reproduced in colour in the online version of *Molecular Membrane Biology*.

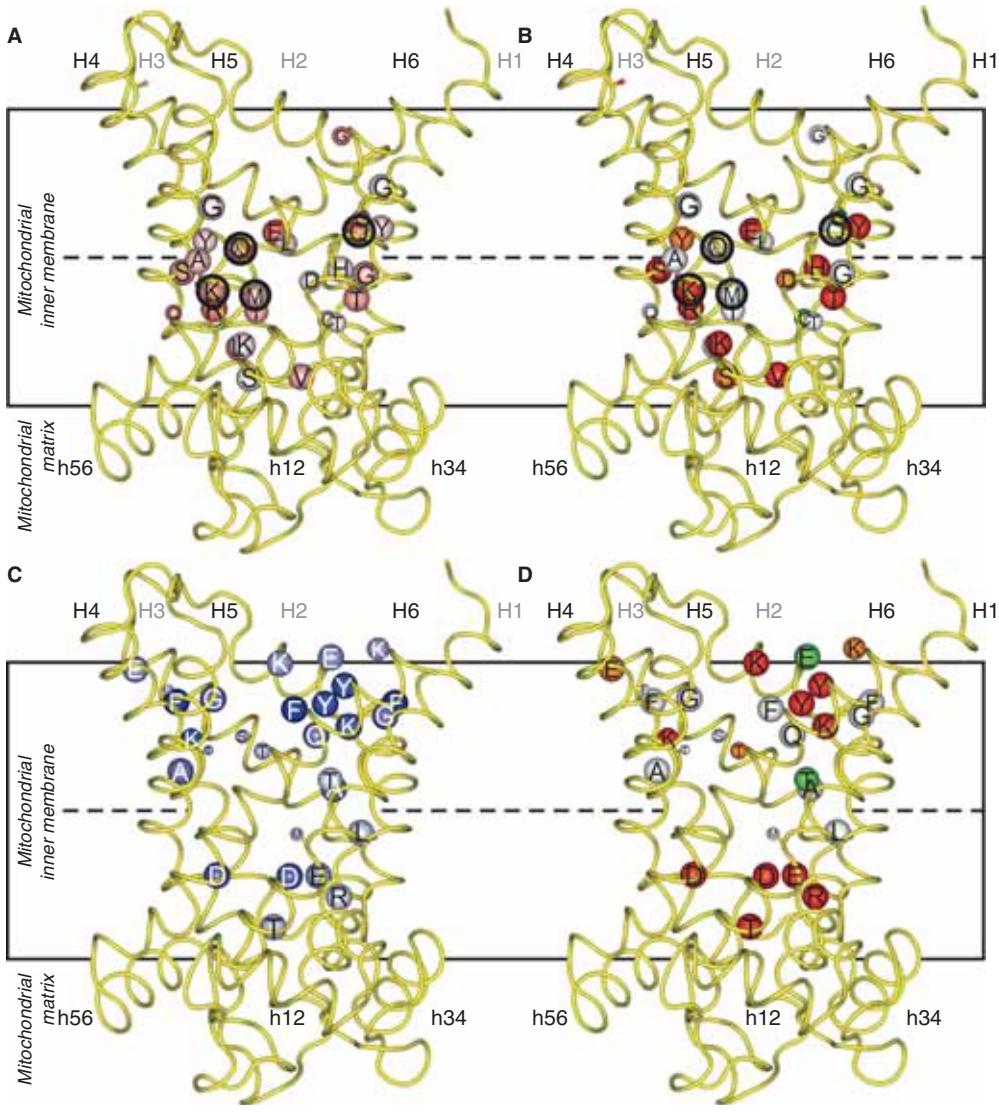

Figure 2. Asymmetry, symmetry and the effects of mutations on transport activity of the yeast phosphate carrier Mir1. Key as in Figure 1. Where the kinetic parameters were measured, the corresponding initial rates were calculated with the Michaelis-Menten equation at a substrate concentration equal to the km of the wild-type. The black encircled residues are the three contact points of the substrate binding site, which in Mir1 consists of Q86, K179, Q180 and M279, all asymmetric as an adaptation to a small substrate. This Figure is reproduced in colour in the online version of *Molecular Membrane Biology*.

conserved and asymmetric residues in the cavity have a clear tendency of clustering in the proposed substrate binding site at a central location in the membrane, whereas the symmetric ones tend to cluster in and around the cytoplasmic and matrix gate (Robinson et al. 2008).

There is a distinct correlation between residues with increasing degree of asymmetry and residues that are critical for transport in OGC (Figure 1A & B), Mir1 (Figure 2A & B) and Ctp1 (Figure 3A & B). The analysis also shows that mutations of the contact point residues are detrimental to transport activity. In Mir1 only contact point II is involved, whereas other interactions are carried out by asymmetric residues, because the substrate is small and requires co-transport with a proton (Kunji and Robinson 2010). However, the residues of the substrate binding site of OGC and Ctp1 are mostly conserved and symmetrical, as a requirement for the coupling of substrate binding to a symmetric transport mechanism. The carriers that transport carboxylic acids have positively charged residues to bind the negatively charged substrates whereas the surrounding asymmetric residues are likely to define the specificity by modulating the immediate environment. For example 2-oxoglutarate carriers, with the combination of RY[TS][RK], RA and [RK] in the three contact points, transport 2-oxoglutarate, L-malate, malonate,

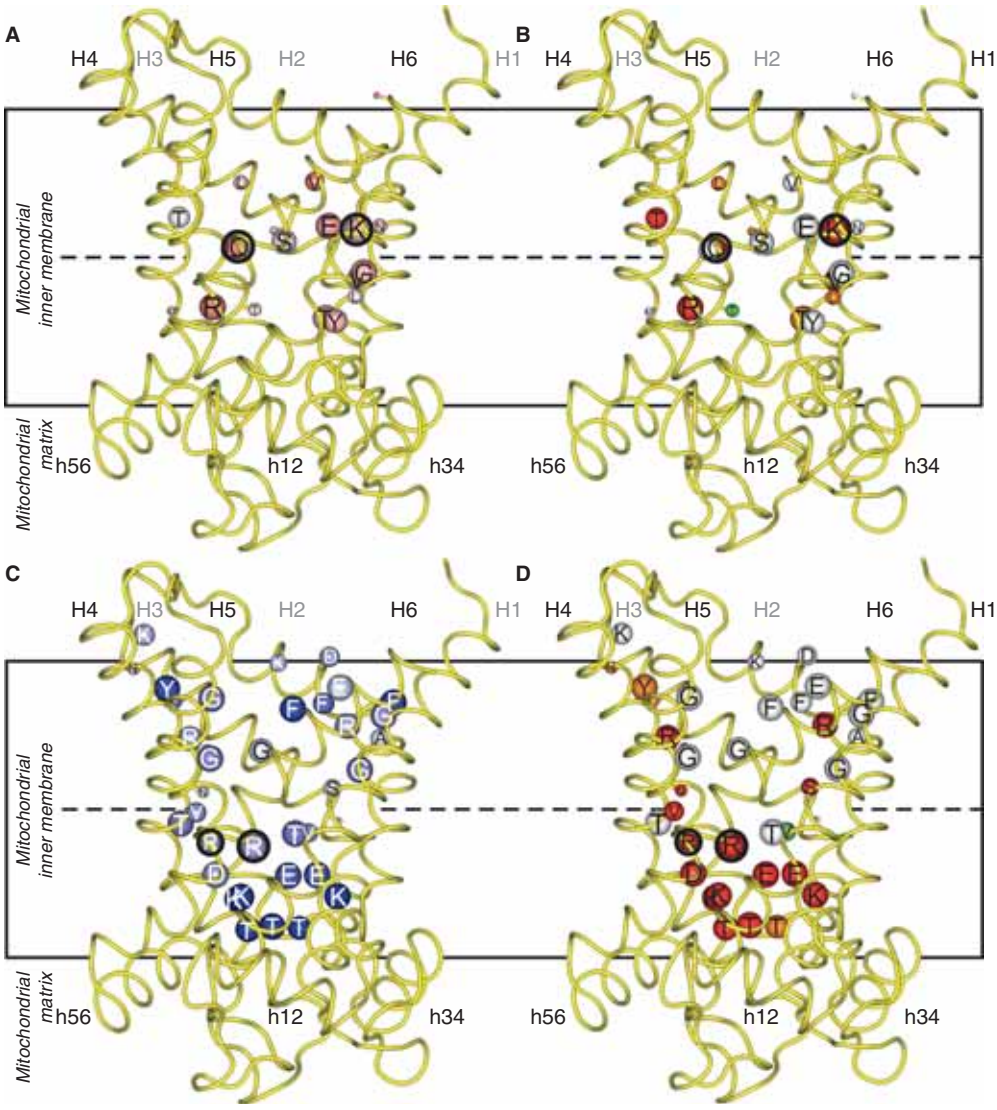

Figure 3. Asymmetry, symmetry and activity of mutants of the yeast citrate carrier Ctp1. Key as in Figure 1. Where the kinetic parameters were measured, the corresponding initial rates were calculated according to the Michaelis-Menten equation at a substrate concentration equal to the km of the wild-type. The black encircled residues are the contact points of the substrate binding site, which in Ctp1 might consist of the conserved and asymmetric K83 and Q182 together with the symmetric R279 and R181. This Figure is reproduced in colour in the online version of *Molecular Membrane Biology*.

maleate, succinate, and to a small extent D-malate and 2-oxoadipate (Bisaccia et al. 1985, Fiermonte et al. 1993) whereas dicarboxylate carriers with RY [ST]R, R[AG] and R in the contact points transport L-malate, phosphate, sulphate, thiosulphate, malonate, maleate and succinate (Palmieri et al. 1996b, Fiermonte et al. 1998). Thus OGC and the dicarboxylate carrier have overlapping, but not identical substrate specificity. Therefore, there must be other determinants that can discriminate between substrates that are similar. Most likely the asymmetric residues close to the contact points take part in this 'fine-tuning' of the substrate specificity (Palmieri et al. 2011).

Mutations that alter substrate specificity

The mutagenesis experiments show that particular residues are important for transport, but this observation does not disclose their role, because they can be directly or indirectly involved in substrate binding, in the structural fold, in the conformational changes during substrate translocation or in the opening and closing of the matrix and cytosoplasmic gates. If a mutation causes an altered substrate specificity then this demonstrates unequivocally that the mutated residue is involved in substrate binding and selection. In our recent paper, the substrate binding site of the two human mitochondrial ornithine carrier

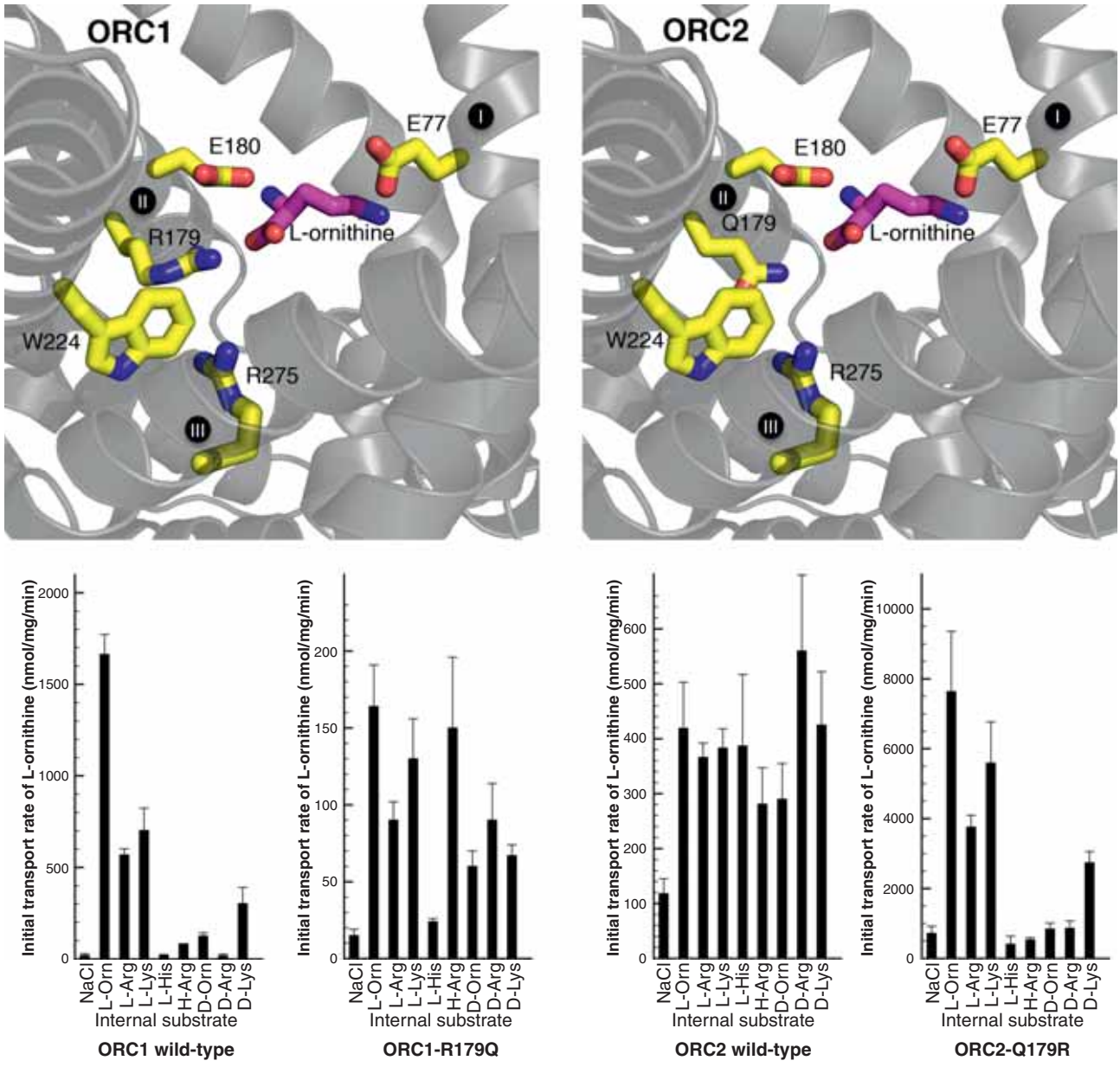

Figure 4. Swapping specificity of the human ornithine carrier by exchanging a single residue in contact point II of the substrate binding site. L-ornithine (magenta) bound in the substrate binding site of ORC1 and ORC2 is shown with the investigated residues (yellow). Results from transport hetero-exchange experiments of radioactive L-ornithine with the wild-type and mutant ORC proteins reconstituted in proteoliposomes, preloaded internally with the various substrates indicated: L-Orn, L-ornithine; L-Arg, L-arginine; L-Lys, L-lysine; L-His, L-histidine; H-Arg, L-homoarginine; D-Orn, D-ornithine; D-Arg, D-arginine and D-Lys, D-lysine (Monné et al. 2012). This Figure is reproduced in colour in the online version of *Molecular Membrane Biology*.

isoforms (ORC1 and ORC2) were probed by site directed mutagenesis of the contact point residues (Monné et al. 2012). The two transporters differ in specificity and transport rate (Fiermonte et al. 2003) and have different residues in contact point II; RE in ORC1 and QE in ORC2. The results demonstrated that this difference is responsible for the difference in substrate specificity between the two isoforms (Figure 4). When the residues are exchanged in the ORC1-R179Q and ORC2-Q179R mutant proteins the substrate specificity and transport rate are swapped as well. Given that mutations in contact point II also affect the turnover number, substrate binding to this residue is a rate limiting step in the catalytic transport cycle. A mutation in the other residue in contact point II ORC1-E180D also displayed changed substrate specificity, indicating that this position is also important in substrate selection.

The most straightforward interpretation is that the C_α carboxylate and amino group of the amino acid substrate bind to R/Q179 and E180 of the ornithine carriers. When bound to this position the only plausible binding site for the terminal amino group is residue E77 in contact point I. This residue is conserved and unique for the ORC subfamily and the results show that it cannot be altered without total loss of function. It was also noted that contact point II might be interacting indirectly with contact point III through cation-π interactions. In this way, substrate binding would engage all three contact points for coupling substrate binding to a symmetrical mechanism.

The R294A mutation of the yeast mitochondrial ADP/ATP carrier Aac2 also alters the substrate specificity, demonstrating that contact point III is also involved in substrate binding (Heidkämper et al. 1996). This mutant protein transports ADP at an almost unchanged rate compared to the wild-type but ATP transport is reduced to less than 10%, suggesting that R294 is important for binding the γ-phosphate of ATP.

Analysis of mutations of residues involved in the transport mechanism

The systematic mutagenesis of OGC has highlighted the structural elements that are conserved, symmetrical and important for transport (Figure 5). Apart from those in the vicinity of the substrate binding site (Figure 5A), critical residues are also found in the conserved signature motifs of the odd- and even-numbered helices that form the matrix and cytoplasmic gates, respectively. Similar to what was observed for the asymmetric residues, it seems that residues with increasing degree of symmetry coincide with positions where mutations are affecting the transport more severely in OGC (Figure 1C & D), Mir1 (Figure 2C & D) and Ctp1 (Figure 3C & D). Here we review these residues to see whether they are likely to be involved in substrate binding or in critical aspects of the transport mechanism or the structure.

The matrix gate mutations include residues of the signature motif PX[DE]XX[RK] that are highly conserved in all carriers and can therefore not have a discriminatory role in substrate recognition (Figure 5B). Carriers transporting NAD^+ (Todisco et al. 2006, Palmieri et al. 2009), pyrimidine nucleotides (Marobbio et al. 2006, Floyd et al. 2007), FAD/folate (Tzagoloff et al. 1996, Titus and Moran, 2000, Bedhomme et al. 2005), and coenzyme A, FAD and NAD^+ in peroxisomes (Agrimi et al. 2012a, 2012b, Bernhardt et al. 2012) have a conserved W instead of [DE] in the second signature motif; FAD/folate carriers and ATP-Mg^{2+}/phosphate carriers (Fiermonte et al. 2004, Traba et al. 2008, 2009) have a glutamine and [QNAT], respectively, instead of the negatively charged residue of the third signature motif; dicarboxylate carriers (Palmieri et al. 1996b, 2008, Fiermonte et al. 1998) have an asparagine or methionine instead of the positively charged residue of the second motif; the fungal oxaloacetate/sulphate/α-isopropylmalate carrier (Palmieri et al. 1999, Marobbio et al. 2008) lacks the negatively charged residue and the positively charged residue in the second and third motifs, the former residue being replaced by [FY] and the latter by [LM]; and the phosphate carriers (Runswick et al. 1987, Dolce et al. 1994, Wohlrab and Briggs 1994) have a hydrophobic substitution instead of the positively charged residue in the third motif. These mostly polar modifications would be capable to either cation-π or hydrogen bond interactions, which have an interaction energy that is approximately half of an ionic bond. There is no straightforward correlation to substrate specificity, since the tryptophan modification, for example, is the same in the NAD^+, pyrimidine nucleotides, FAD/folate and peroxisomal coenzyme A/FAD/NAD^+ transporters (see above), but their substrates are very different in chemistry and biophysical properties. It is possible that the strength of the matrix network is modulated to be lower than the interaction energy of the substrate with the binding site. Just below the matrix salt bridge network are conserved and symmetrical glutamines, which could form a hydrogen bond with residues involved in the matrix salt bridge network, but there is no correlation to substrate specificity (Figure 5B). The positively charged residue that follows the matrix salt bridge network residues interacts with a negatively charged residue of the [DE]G motif and conserved Y, linking the matrix α-helices to the odd-numbered α-helices, but they are outside of the central cavity and occluded (Figure 5D). The residues at the cytoplasmic gate in and around the motif [YF]XX[YF] and [DE]XX[RK] are (Figure 5C), although less conserved, found in a wide range of carriers with different substrates and are therefore also unlikely to form a basis for substrate specificity. There are also many conserved and symmetric glycines, of which some are found in the GXXXG motif below the cytoplasmic gate in the odd-numbered helices that have been shown to be crucial for transport (Figure 5B). There are two separate hypotheses to describe their role in carrier activity: (i) They could form important pivot points for close helix-helix interactions (Melnyk et al. 2004, Robinson et al. 2008), or (ii) they could form hinges in the helices

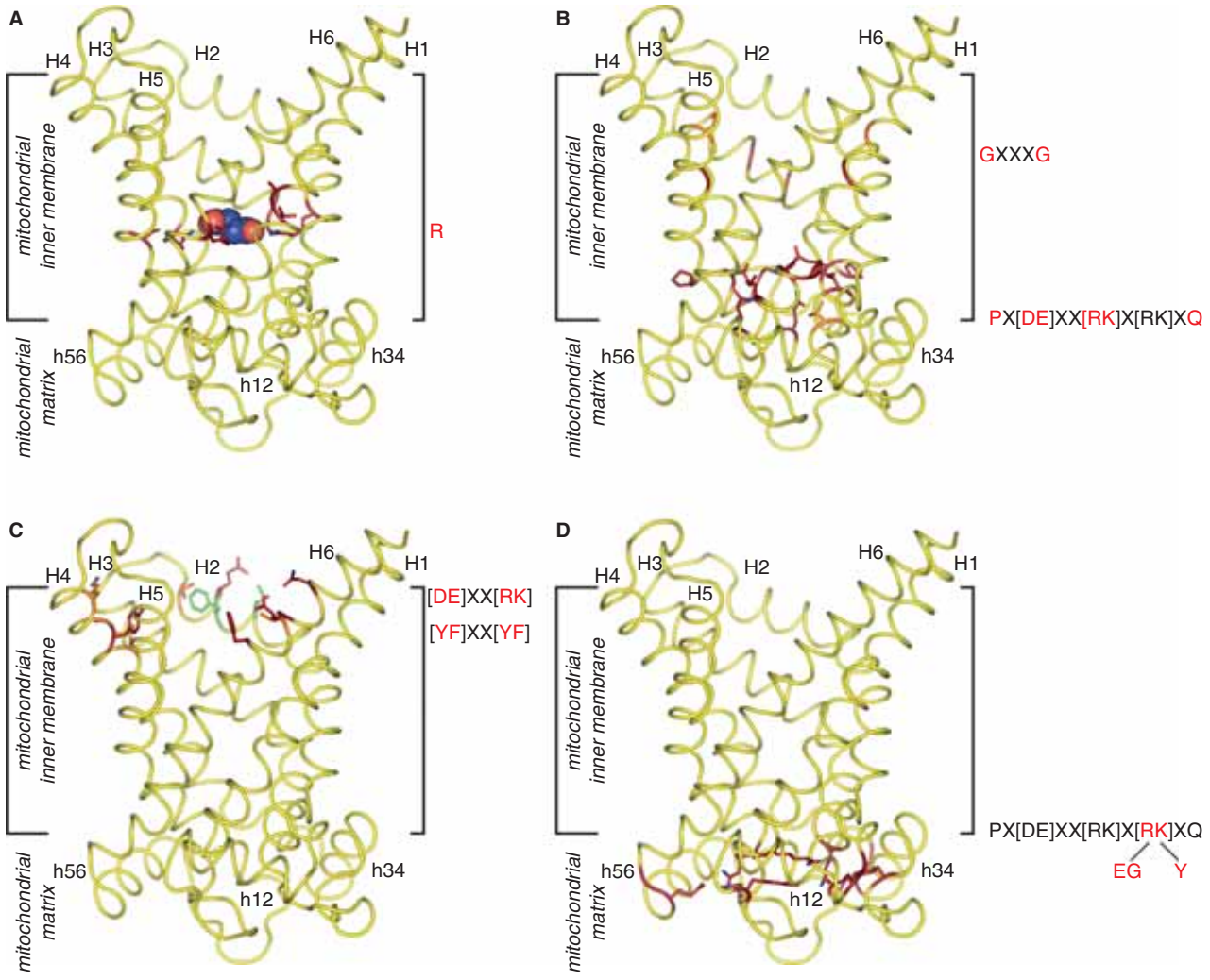

Figure 5. Conserved and symmetrical residues that are critical for function of the mitochondrial oxoglutarate carrier. (A) Residues of the substrate binding site, including the symmetrical and conserved arginine triplet, which are the contact points. (B) Triplets of symmetry-related residues belonging to the GXXXG motif and the PX[DE]XX[RK] motif, which contain a conserved P that is present at the kink and the conserved charge residues of the matrix salt bridge network, which forms in the cytoplasmic state. Underneath are the conserved and symmetrical Q residues, which may interact with the network. (C) Triplets belonging to the aromatic motif [YF]XX[YF] and the cytoplasmic salt bridge network [DE]XX[RK], which are present on the even-numbered α-helices at the cytoplasmic side of the carrier. The charged residues may form a network when the carrier is in the matrix-state. (D) Conserved positively charged [RK] residues, which follow the matrix network and interact with the negatively charged residue of the [ED]G motif. The Y may also be involved in the bonding arrangement, linking the matrix α-helices to the odd-numbered α -helices. The model of OGC, based on the structure of the bovine ADP/ATP carrier, is shown in yellow and the aforementioned residues are shown in red when they are critical to the function, orange if they are important and green when they are not important. This Figure is reproduced in colour in the online version of *Molecular Membrane Biology*.

necessary for the opening and closing of the gates (Palmieri and Pierri 2010).

Thus it is unlikely that the critical residues of the matrix and cytoplasmic gates could be involved in substrate binding and it is much more likely that they are involved in the closing and opening of the gates in the transport cycle. Since they are in the translocation pathway, it is also possible that they modulate or facilitate the entry of substrates to the central substrate binding site or the exclusion of others.

Conclusions/perspectives

In this review we have shown that the conclusions drawn from theoretical and experimental approaches to define the substrate binding site in mitochondrial carriers largely agree. In fact, the sequence/structure analysis complements interpretation of the mutant data and vice versa. The approach of combining site-directed mutagenesis and transport assays with a set of substrates has proved to be successful for

determining the residues that are directly involved in substrate interactions. Taken together, the mutagenesis studies of well-characterized carriers have validated the importance of specific residues in substrate binding and transport mechanism. Furthermore, the identification of contact point residues in the mitochondrial carrier substrate-binding site has helped and will continue to be useful in explaining differences in half-saturation constants of substrates for specific carriers (Marobbio et al. 2008) and in predicting the substrates, or at least the class of substrates, that are transported by yet uncharacterized mitochondrial carriers (Castegna et al. 2010, Palmieri et al. 2011, Stael et al. 2011).

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