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The conserved substrate binding site of mitochondrial carriers

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Abstract

Mitochondrial carriers transport nucleotides, co-factors and metabolic intermediates across the inner mitochondrial membrane permeability barrier. They belong to a family of transporters unique to eukaryotes and they differ in structure and transport mechanism from other secondary transporters. The main structural fold consists of a barrel of six transmembrane α-helices closed at the matrix side by a salt-bridge network at the bottom of the cavity. The significant sequence conservation in the mitochondrial carrier family suggests that specific recognition of substrates is coupled to a common mechanism of transport. We have identified a common substrate binding site comprising residues that are highly conserved and, as demonstrated by mutagenesis, are essential for function. The binding site explains substrate selectivity, ion coupling and the effects of the membrane potential on transport. The main contact points in the site are related by threefold symmetry like the common structural fold. The substrate is bound at the midpoint of the membrane and may function as a pivot point for the movements of the transmembrane α-helices as the carrier changes conformation. The trigger for the translocation event is likely to be the substrate-induced perturbation of the salt bridge network at the bottom of the cavity.

Keywords: Mitochondrial carrier; Substrate binding; Transporter; Bioinformatics; Structure

1. Introduction

Members of the mitochondrial carrier family link cytosolic and mitochondrial metabolism by facilitating transport across the inner mitochondrial membrane. The substrates of the transporters include nucleotides, amino acids, co-factors, carboxylic acids and inorganic anions. These metabolites are required for processes including oxidative phosphorylation, gluconeogenesis, the synthesis and degradation of amino and fatty acids, the macromolecular synthesis of proteins and nucleic acids, and thermogenesis [1,2]. The first amino acid sequences of mitochondrial carriers [3–7] clearly demonstrated that the carriers were structurally related [6]. Sequence analyses indicated that the carriers have three homologous sequence repeats of about one hundred amino acid residues each, as was noted first in the published sequence of the bovine ADP/ATP carrier [8]. The carriers also have a signature motif P-X-[DE]-X-X-[RK], which is highly conserved in all members of the family and in all three repeats (PROSITE PS50920 and PFAM PF00153).

The atomic model of the bovine mitochondrial ADP/ATP carrier (BtAAC1) has explained these sequence features in molecular detail [9]. The main structural fold is a bundle of six α-helices with threefold pseudo symmetry (Fig. 1). The prolines of the signature motif induce a sharp kink in the odd-numbered α-helices, which close the central pore of the α-helical bundle on the matrix side. These α-helices are held together by salt-bridges that are formed between the charged residues of the three signature motifs, and a re-arrangement of these bonds would open the carrier to the mitochondrial matrix side (Fig. 1) [10].

The structure also provided detailed information on how the inhibitor carboxy-atractyloside (CATR) is bound in the open cavity formed by the α-helical bundle [9]. The inhibitor is not an obvious structural analogue of ADP because it is larger and does not have the same functional groups. This means that the nucleotide binding site has not been defined structurally. Mutagenesis has shown that some of the amino acid residues involved in the binding of CATR are essential for adenine nucleotide transport, but these residues could be involved in any part of

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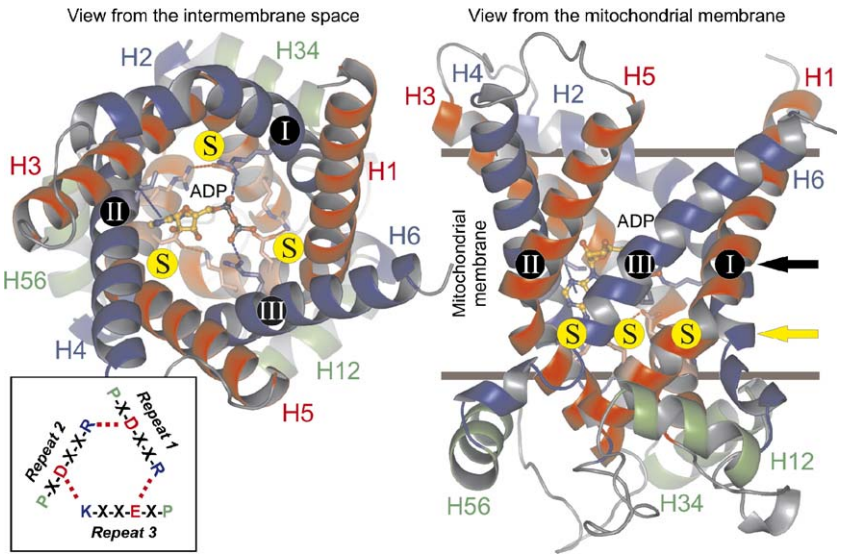

Fig. 1. Threefold pseudo three-fold symmetry of the ADP/ATP carrier, the salt bridge network and the substrate binding site. Structure of the bovine ADP/ATP carrier BtAAC1 viewed from the intermembrane space (A) and the membrane (B). The coordinates for the structure were obtained from PDB entry 1okc [9]. The odd-numbered, even-numbered and matrix α-helices are coloured in red, blue and green, respectively. The salt bridge network at the bottom of the cavity is indicated by the letter S and by red dotted lines between positively and negatively amino acid residues of the signature motif. Also shown is a schematic representation of the salt-bridge network at the bottom of the cavity. An ADP molecule is bound to the proposed substrate binding site at contact points I, II, and III [11].

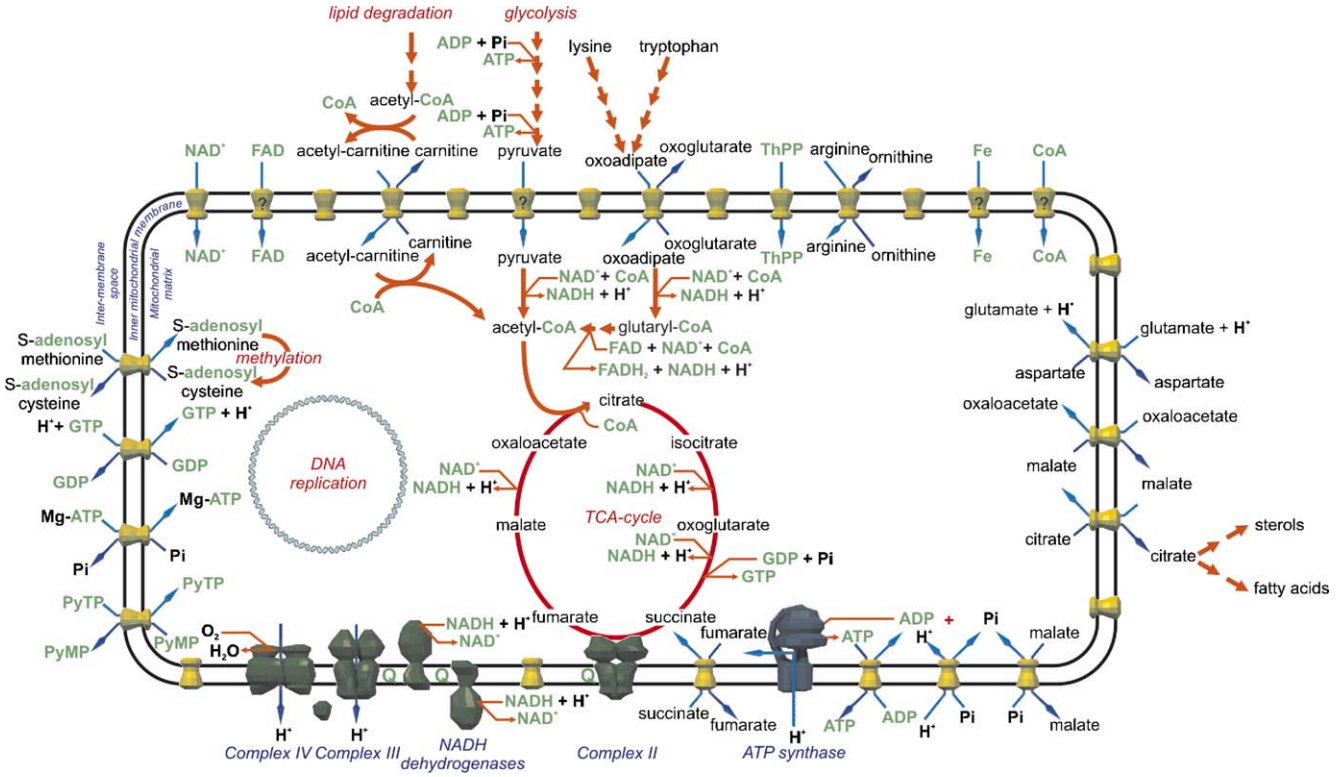

Fig. 2. Role of yeast mitochondrial carriers in mitochondrial and cellular metabolism. Schematic representation of the yeast mitochondrial inner membrane, showing ATP synthase, the complexes of the respiratory chain and mitochondrial carriers. The outer mitochondrial membrane is not shown. Red arrows indicate the metabolic pathways, which are simplified to emphasise key metabolites. Co-factors, high-energy intermediates and reducing equivalents are indicated in green. Note that the same metabolites appear at different sites, indicating that the corresponding pathways are linked. Blue arrows show the directionality of the transport steps over the inner mitochondrial membrane as carried out by the membrane proteins. The proposed transporters of CoA, FAD, iron and pyruvate are marked with a question mark, because the substrate has not been confirmed by transport assays with purified and reconstituted protein. The references that describe the assignment of functions are given in the main text. Only one isoform of the identified carriers is shown.

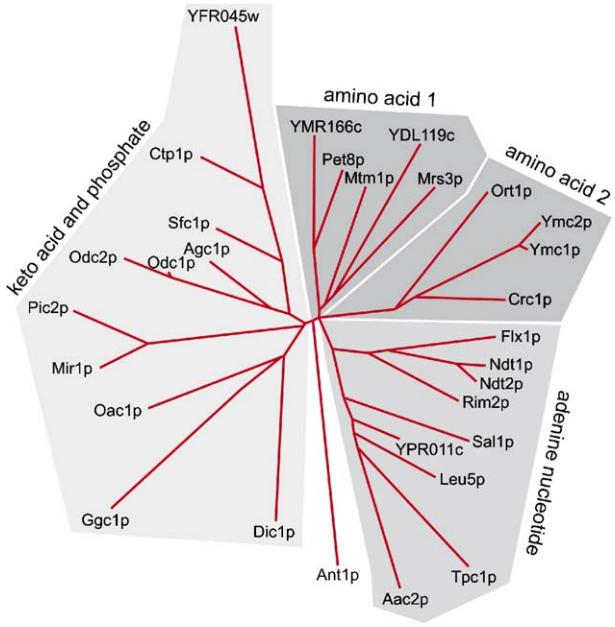

Fig. 3. The unrooted phylogenetic tree of the mitochondrial carrier family in *Saccharomyces cerevisiae*. The phylogenetic tree was calculated from ungapped multiple protein sequence alignments of the six transmembrane α-helices using maximum likelihood implemented in the application ProML of the PHYLIP3.6 package [61]. The JTT substitution matrix was used for the exchangeability parameters of amino acids, with a gamma correction to allow for rate heterogeneity [62]. The alpha parameter of the gamma distribution was varied systematically to find an optimal value. The Aac1p and Aac3p isoforms of the ADP/ATP carrier Aac2p are omitted as is Mrs4p, a homologue of Mrs3p. The yeast proteins YDR470c and YHM2 were excluded from the phylogenetic analysis.

the transport cycle. The binding of CATR and ADP is mutually exclusive [9], indicating that the binding sites probably overlap.

The ADP/ATP carrier binds CATR when the substrate binding site for ADP is open to the mitochondrial intermembrane space and the structure agrees with this notion. However, what stage in the transport cycle has been captured? As CATR could be binding and distorting the binding site of ADP, the structure may not resemble the empty state of the carrier. It may also not represent the nucleotide-binding state, because this should have led to a conformational change to a state that is open to the matrix side. In this review, we will describe series of steps that have led to our hypothesis for substrate recognition and binding [11]. Our basic approach was to correlate well-defined substrate specificity [12–29] to a binding site in structural models of the carriers based on the bovine ADP/ATP carrier [9]. The common substrate binding site for mitochondrial carriers is supported by amino acid conservation and experimental evidence.

1.1. The substrate specificity of yeast mitochondrial carriers

The characteristic sequence features of mitochondrial carriers provide the means to search for genes that encode mitochondrial carriers in genome sequences. The total number of mitochondrial carriers found in genomes correlates roughly with the extent of the biosynthetic capability of the organism. Eukaryotic parasites that harvest metabolites from the host cell have a small number of carriers (1 to 9), whereas plants, which have a relatively large biosynthetic capability, have close to 60. The transport properties of yeast and human mitochondrial carriers have been well characterised [1,30]. The most successful strategy for identification of the function of carriers is based on the over-expression of the carrier in inclusion bodies in *E. coli*,

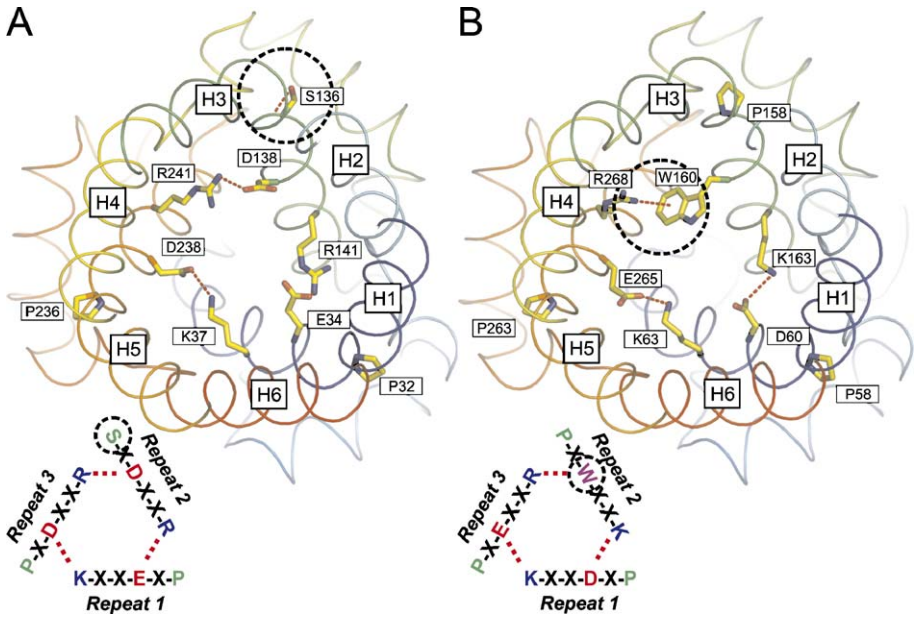

Fig. 4. Models of the salt bridge network formed by the signature motifs of the yeast ADP/ATP carrier Aac3p (A) and the NAD^+ carrier Ndt2p (B). The structural folds of Aac3p [28] and Ndt2p [12] are shown as tubes and are viewed from the intermembrane space. The interacting side chains of the signature motif are shown in stick representations. Also shown is a schematic representation of the salt bridge network with the anomalies in the signature motif encircled by a dotted line.

followed by refolding, reconstitution into liposomes and transport assays [31]. Other approaches use the functional expression of mitochondrial carriers in yeast mitochondria [24] or in the cytoplasmic membrane of the Gram-positive bacterium *Lactococcus lactis* [32].

The starting point for our analysis was the yeast mitochondrial carrier proteome (Fig. 2). Out of the 35 carriers encoded by the nuclear genome, 22 have been identified in transport assays by using purified reconstituted protein. They are three isoforms of the ADP/ATP carrier (Aac1p, Aac2p and Aac3p) [28,29], the aspartate–glutamate carrier (Agc1p) [18], the peroxisomal adenine nucleotide transporter (Ant1p) [21], the carnitine carrier (Crc1p) [24], the citrate transport protein (Ctp1p) [33], the dicarboxylate carrier (Dic1p) [34], the GDP/GTP carrier (Ggc1p) [15], two isoforms of the phosphate carrier (Mir1p and Pic2p) [20,35], the oxaloacetate carrier (Oac1p) [23], two isoforms of the oxodicarboxylate carrier (Odc1p and Odc2p) [22], the ornithine transporter (Ort1p) [26], the S-adenosyl methionine (SAM) transporter (Pet8p) [17], the Mg^{2+}–ATP/P_i carrier (Sal1p) [14,16], the succinate–fumarate carrier (Sfc1p) [25], the pyrimidine transporter (Rim2p) [13], the thiamine pyrophosphate carrier (Tpc1p) [19], and two isoforms of the NAD^+ transporter (Ndt1p and Ndt2p) [12]. There are four carriers for which clues for a substrate have been obtained from genetic studies and accumulation of compounds in mitochondria, namely the FAD carrier [36], the CoA transporter [37], and the two isoforms Mrs3p and Mrs4p that are involved in iron accumulation [38,39]. The yeast carrier YIL006w has been proposed to be the pyruvate carrier [40], but transport studies have shown that it is one of the two isoforms of the NAD^+ carrier [12]. Therefore, the gene for the pyruvate carrier remains to be identified and is possibly among the nine yeast mitochondrial carriers without assigned functions.

1.2. Classification of the yeast mitochondrial carriers by substrate and phylogeny

The substrates that are transported by the members of the yeast mitochondrial carrier family vary in size, functional groups and charge. On the basis of the structure of the substrates, the proteins were classified into three major subfamilies; (i) transporters of amino acids (Agc1p, Crc1p, Ort1p and Pet8p), (ii) carriers of keto acids (Ctp1p, Dic1p, Oac1p, Odc1p, Odc2p, and Sfc1p), and (iii) transporters of adenine nucleotides (Aac1p, Aac2p, Aac3p, Ant1p, Pet8p, Sal1p, Ntd1p and Ndt2p). As the substrate *S*-adenosyl methionine contained both a zwitterion and an adenine moiety, the carrier Pet8p was placed in two subfamilies initially.

The phylogenetic tree of yeast mitochondrial carriers shows four major clades, consistent with the classification based on substrate structure (Fig. 3). One clade consists of the adenine nucleotide transporters, the second of keto acid and phosphate carriers and the remaining two clades contain the amino acid carriers. The peroxisomal ATP/AMP carrier Ant1p forms a separate group from the mitochondrial carriers. Ggc1p, Mir1p and Pic2p cluster with members of the keto acid transporter subfamily, Tpc1p and Rim2p with the adenine nucleotide subfamily and Agc1p with the keto acid rather than the amino acid carriers.

1.3. Comparative models of the yeast mitochondrial carriers

In order to study substrate binding sites, comparative models were built by using sequence alignments and the structure of the bovine ADP/ATP carrier as a template [9]. Despite the fact that the carriers have relatively low sequence identity, there is very little ambiguity in the alignments because of the threefold repeats, signature motifs and other highly conserved amino acid residues. The transmembrane α-helices are also strongly amphipathic, with one face of each α-helix interacting with the lipid bilayer and the other facing the water-filled cavity. Often, the residues in the cavity are highly conserved compared with those interacting with the lipid bilayer and others in the loop regions. These properties provided additional guides for the amino acid sequence alignments.

Residues in the signature motif P-X-[DE]-X-X-[KR] were not absolutely conserved. Firstly, proline is replaced by serine in YMR166c (S73), Aac1p (S138), Aac2p (S147) and Aac3p (S136), and so its C_β O was hydrogen-bonded to the main chain to mimic proline (Fig. 4A). Secondly, the signature motifs of the first repeat in Flx1p, Rim2p, YEL006w and YIL006w contain tryptophan instead of the expected acidic residue, but they have

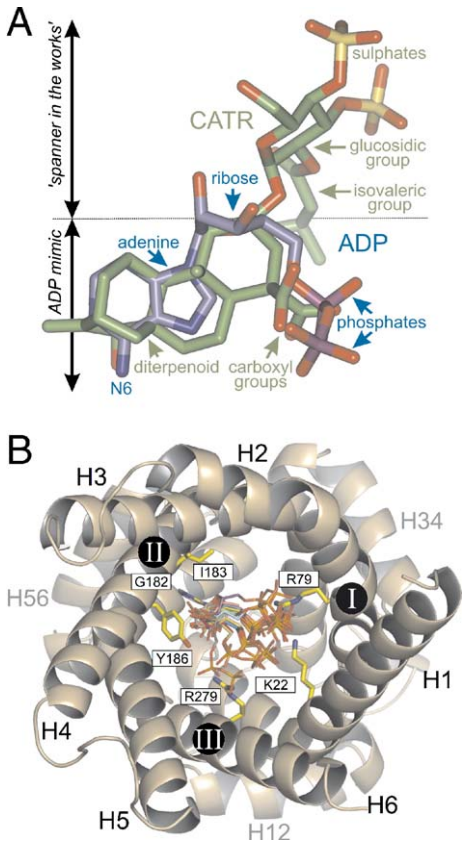

Fig. 5. Superposition of CATR and ADP (A) and different conformations of ADP in the substrate binding site (B). (A) Carbon atoms of CATR and ADP are shown in green and in blue, respectively. Oxygen atoms, sulphur atoms and phosphorous atoms in red, yellow and purple, respectively. (B) Representation of the bovine ADP/ATP carrier BtAAC1 (1 OKC) [9] with ADP docked in the substrate binding site in different conformations (from PDB entries 1g99, 1a9x, 1rfu, 1ty8, 1e3m, 1w0j,1e3m,1woj, 1nne, 1oz4, and 1m7h). Amino acid residues involved in the binding of ADP are indicated in stick representations.

retained the basic residue, and this interaction was modelled as a cation–π interaction [41] (Fig. 4B).

1.4. The substrate binding site of keto and amino acid transporters

Amino acid and keto acid substrates are relatively small and often they possess the same functional groups. For example, the 2-oxoglutarate substrate of Odc1p is identical to glutamate, the substrate of Agc1p, except for having an amino rather than a keto group on the C2 carbon. The Ort1p substrate arginine and Agc1p substrate glutamate are both amino acids but they differ in the functional group of the side chain, i.e., carboxyl versus amino. This means that the substrate binding sites of carriers must be able to recognise all functional groups simultaneously in order to discriminate between the various substrates. In other words, the substrate binding site must have several residues of compatible chemistry in the correct spatial geometry to bind the substrate. The side chains in the water-filled cavity were the most likely candidates for the interactions with the substrate. The peptide units of the backbone are unlikely to be involved in substrate binding, because they participate in hydrogen bonding required for the structural integrity of the α-helices. The side chains interacting with the lipids were not considered because the substrates are charged and cannot bind to the protein–lipid interface. Finally, the loops regions were excluded as they are relatively poorly conserved and their lengths differ.

The search for the substrate-binding site in the cavity used two major constraints based on the principles formulated above. The first constraint was that the chemical properties of the functional groups had to be compatible with substrate binding. For example, carboxyl groups of the substrate could bind to polar and positively charged, but not to apolar or negatively charged side chains. The second constraint was that all side

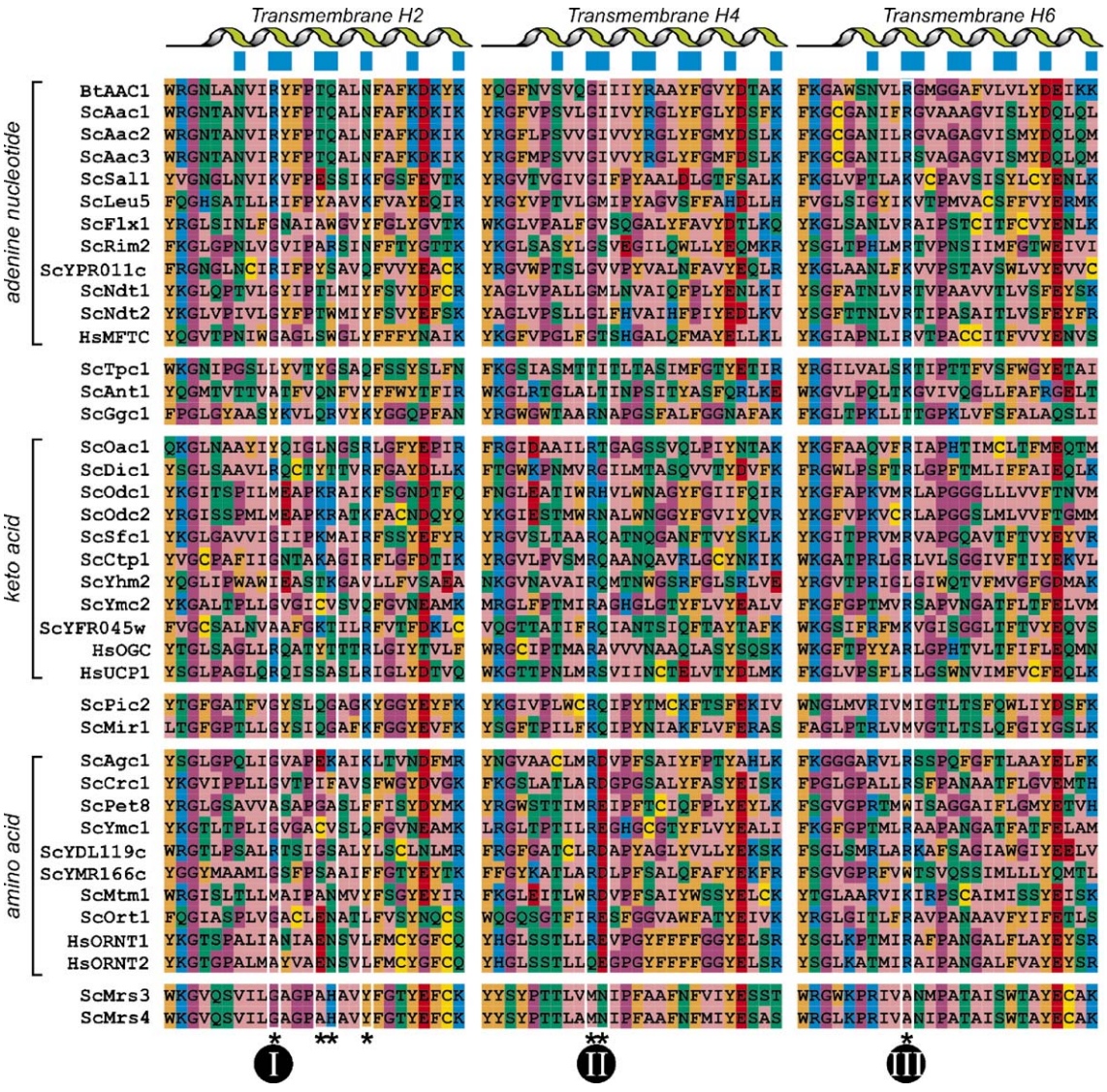

Fig. 6. Amino acid sequence alignment of the even numbered transmembrane α-helices of yeast and human mitochondrial carriers grouped by substrate type. The blue boxes above the alignment indicate the residues that face the cavity. The alignment is in the ZAPPO colouring scheme. The three contact points are indicated by asterisks and by a white box around the relevant residues. The abbreviations Bt stand for bovine (*Bos taurus*), Hs for human (*Homo sapiens*), and Sc for yeast (*Saccharomyces cerevisiae*) carriers.

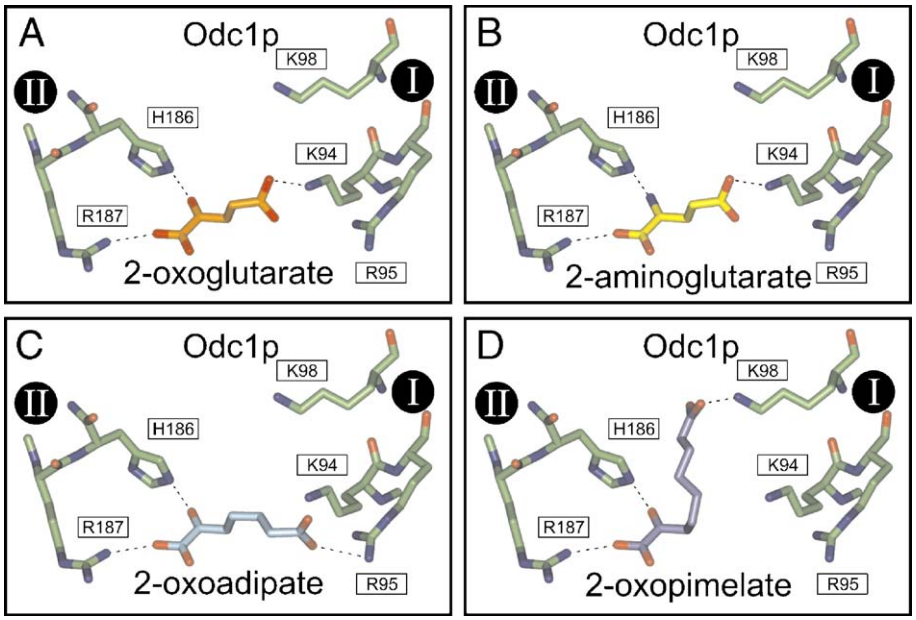

Fig. 7. Overview of the substrate binding site of the oxodicarboxylate transporter with different substrates. The natural substrates of the oxodicarboxylate carrier Odc1p [22] are docked in the binding site, i.e., 2-oxoglutarate (A), 2-aminoglutarate (B), 2-oxoadipate (C) and 2-oxopimelate (D). Contact point III that interacts with the carboxyl group of the common functional group has been omitted.

chains involved in binding had to be within the maximal distances of all of the functional groups of the substrate. The distance constraint was not applied too strictly to allow for deviations in the atomic positions in the structure of the bovine BtAAC1 structure demonstrated by high temperature factors, for different conformers of the substrate and side chains, and for potential errors in modelling. Thus, a set of residues was excluded as a possible binding site only if they were clearly too far apart to bind all of the functional groups simultaneously. A search of the cavities of the amino and keto acid transporters based on these two criteria revealed hundreds of potential binding sites in each carrier.

The sequence conservation shows that the structures of mitochondrial carriers are conserved. All of them have a salt bridge network at the bottom of the cavity that must rearrange to allow a substrate to pass. Therefore, the specific recognition of a substrate must be coupled to a common structural mechanism of translocation. The most logical and practical way to satisfy this requirement is by having a unique substrate binding site in a common position. This concept provided a third constraint. All of the possible binding sites in the amino and keto acid carriers were examined to identify those that were in a similar position in the structure. Since the functional groups on the amino and keto acid moieties are very close, the complementary side chains interacting with these functional groups had to be close in distance also. The specific functional groups were allowed to bind to an area in the cavity, because their positions were less well defined as they can adopt a large number of conformations. In all of the amino and keto acid carriers, only 56 potential common binding sites were identified.

Among them only six different residue pairs were capable of binding the functional groups of the amino or keto acid moieties, i.e., residues 182/183, 182/235, 32/235, 137/235, 190/183 and 279/235 in the equivalent positions in the bovine ADP/ATP carrier.

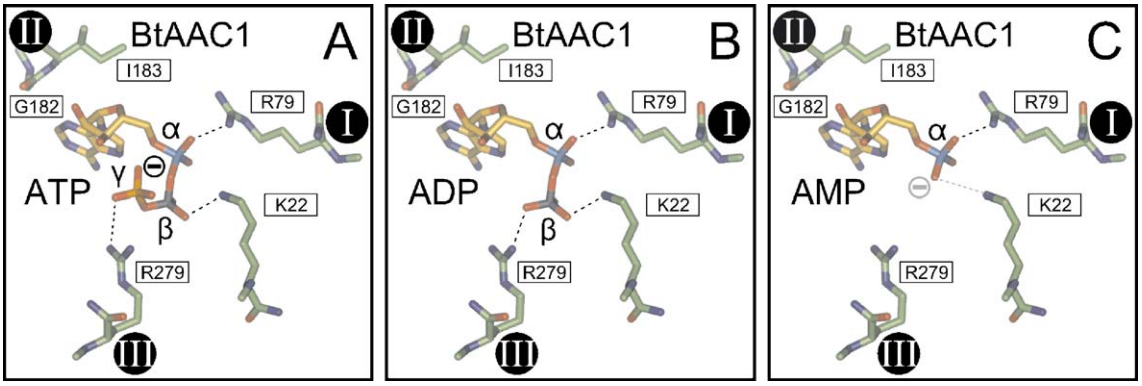

Fig. 8. Binding of adenine nucleotides to the substrate binding site in the bovine ADP/ATP carrier BtAAC1. The coordination of ATP (A), ADP (B) and AMP (C) are depicted in stick representations. The coordinates for the structure of the ADP/ATP carrier were obtained from PDB entry 1 OKC [9].

The fourth constraint was that the site should be able to discriminate between keto and amino acids. Four of the six pairs had polar residues in the positions that interacted with either the keto or the amino group and thus they could not discriminate between these two types of substrates, leaving 182/183 and 190/183 as the only two possibilities. Pair 190/183 was rejected because steric hindrance from the intervening residues prevented both functional groups binding simultaneously. Thus, 182/183 was the only combination capable of binding and discriminating keto and amino groups. There were only three potential areas for binding the specific functional group when the common functional group was bound to 182/183, namely in the neighbourhood of residues 83, 118, and 194. The residues in the area of 118 and 194 were polar and could not discriminate between positively and negatively charged side chains required for the discrimination between arginine and glutamate. Thus, residues in the area of residue 83 were the only ones capable of discriminating between different types of amino and keto acids.

1.5. The substrate binding site of adenine nucleotide transporters

Because adenine nucleotides are substantially larger with more functional groups and a larger number of possible conformations [42], it was not possible to apply the same strategy to identify the substrate binding site of this class of transporters. A large number of ADP and ATP binding sites in the Protein Data Bank [43] was inspected to deduce common chemical and physical properties of adenine nucleotide binding. Although the database is biased towards the P-loop motif [44], the most common nucleotide binding fold, the general consensus was that adenine binds in a pocket flanked by hydrophobic residues Ile, Leu, Val or Met and that phosphates bind to the positively charged residues Arg and Lys. In the water-filled cavity of the ADP/ATP carrier there are very few hydrophobic amino acid residues and the diterpenoid moiety of the inhibitor CATR binds to a hydrophobic pocket created by Y186, I183 and G182. Superposition of CATR and ADP shows that the adenine ring follows the contours of the diterpenoid bicyclo-cis-decalin ring quite well (Fig. 5A). The N1 of adenine is in the position of the diterpenoid ethylene group and the N6 in the position of the diterpenoid hydroxyl group, placing it close to the salt bridges. The phosphates of the substrate are close to the two carboxyl groups of the inhibitor. The photo-activated ADP analogue 2-azido[α-^{32}P]adenosine diphosphate reacts with I183, supporting this assignment [45]. ADP molecules were docked in various conformations in the site (Fig. 5B). Y186 guides the ribose and phosphates to three positively charged residues at the opposite side of the cavity to R79, K22, and R279 (Fig. 5B). Thus, the diterpenoid moiety and the carboxylic groups of CATR mimic ADP, which draws the inhibitor to the substrate binding site. The glucosidic, isovaleric and sulphate groups of CATR act as a 'spanner in the works' by preventing the progression to the alternative state by binding tightly to the carrier. Thus, the structure can be interpreted as a trapped early to intermediate stage in the substrate import cycle, during which the substrate/inhibitor is bound to the binding site, but with the carrier prevented from progressing to the state that is accessible from the mitochondrial matrix.

Another discovery was that the residues involved in adenine binding are in the same position as those involved in binding the common functional groups of keto acids or amino acids. Residue R79 involved in binding the phosphate is in the contact point for the specific group that defines the type of keto acid or amino acid. The third contact point at position R279 is involved in binding of phosphate. There is an equivalent residue, either Lys or Arg in the keto acid or amino acid transporters, which does not confer specificity but it could be involved in binding the common carboxylate groups. Strikingly, the three contact points are related in position by threefold pseudo symmetry, reflecting the structural fold of the carrier. The amino acid residues involved in substrate recognition are on

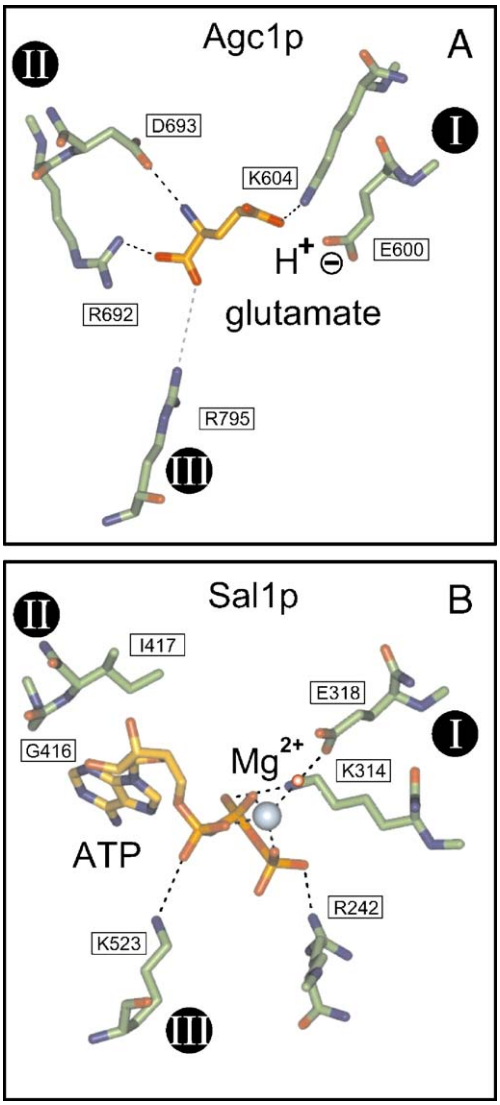

Fig. 9. Ion coupling in the aspartate/glutamate carrier and the Mg^{2+}–ATP/Pi carrier. The binding site for the aspartate/glutamate carrier Agc1p [18] (A) and the Mg^{2+}–ATP/Pi carrier Sal1p [14,16] (B). The interacting side chains and substrates are depicted in stick representations and Mg^{2+} as a sphere. The coordination of the Mg^{2+} ion may require the involvement of a water molecule (small sphere).

H2, H4 and H6, and the corresponding contact points are named I, II and III.

1.6. Conservation of the substrate contact points

The most striking correlation between substrate class and conservation of amino acids is found at contact point II on H4, where all amino acid carriers possess the motif R-[DE], all nucleotide carriers have G-[IVLM], and all keto acid carriers R-[QHNT] (Fig. 6). Thus, the residues at contact point II recognise and discriminate between the different classes of substrates. The phosphate carriers have the binding site of keto acid transporters and they cluster together in one clade (Fig. 3). The dicarboxylate carrier Dic1p transports dicarboxylates as well as phosphate, which would be in agreement with this notion.

The residues at contact point I discriminate between different substrates of the same class. Carboxyl or phosphate groups bind to [RK], amino groups to [DE], aromatic moieties stack with [FY], and hydrophobic moieties such as acyl or hydrophobic side chains form van der Waals interactions with [ILV]. The residues at contact point III are usually [RK] and they bind either carboxyl or phosphate groups. Occasionally the residues in the odd numbered α-helices are also involved in substrate binding, but they are always at a similar height in the protein with respect to the membrane plane as the three contact points. Examples are residue K22 in the bovine ADP/ATP carrier (Fig. 8B), residue R242 in the Mg^{2+}–ATP carrier (Fig. 9B), and residue E105 in the S-adenosyl methionine carrier [11,17]. These additional interactions may improve substrate selectivity and accomplish substrate charge neutralisation.

The residues involved in substrate binding are always well-conserved in homologous carriers from different organisms with the same function [11]. Also, residues that have been mutated are essential for function [9,46–50]. The proposed substrate binding site always has the correct chemistry and geometry for binding of the substrate, but because of the potential error in modelling, the exact conformations of the substrate and side chains cannot be determined.

1.7. General properties of the substrate binding site

Mitochondrial carriers usually exchange structurally related compounds, for example ADP for ATP and malate for 2-oxoglutarate. Often, the exchanged compounds are the start and end product of conversions, oxidation or transamination events in the mitochondrial matrix and the cytoplasm. In intact mitochondria, substrate concentration gradients and the membrane potential ensure that carriers translocate substrates in one

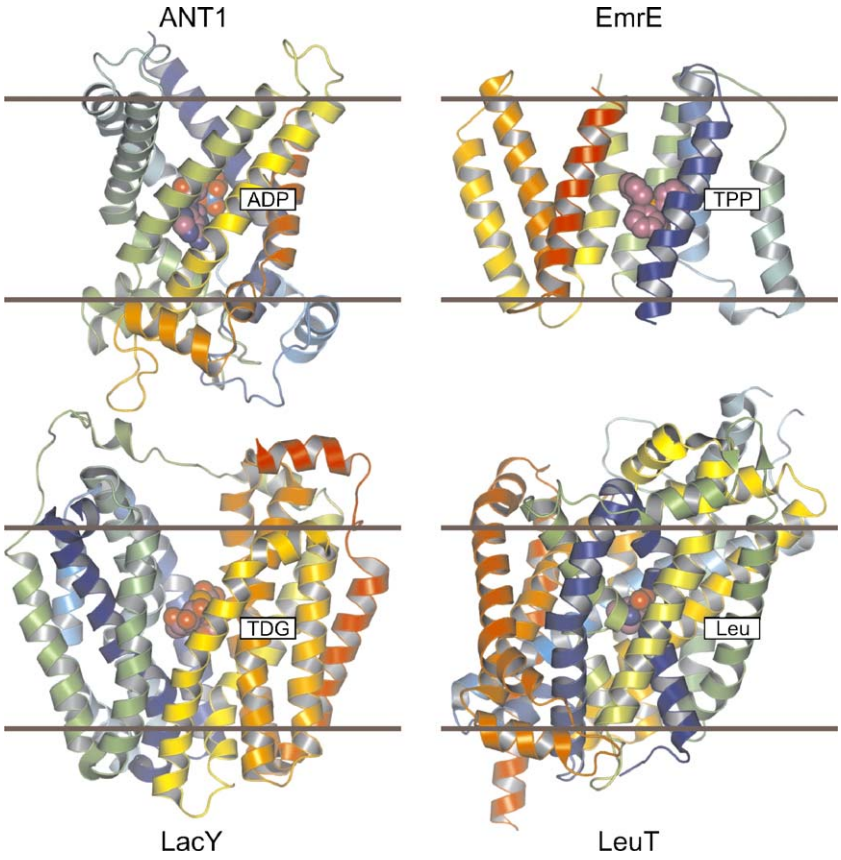

Fig. 10. Structures of transporters of small organic molecules. The approximate borders of the membrane are indicated by purple lines based on the occurrence of hydrophobic and aromatic residues. The main structural fold is depicted in cartoon representation coloured in a gradient from blue (amino-terminus) to red (carboxy-terminus). The substrates and substrate analogues, ADP (A), tetraphenyl-phosphonium (TPP) (B), and thiodigalactoside (TDG) (C) and leucine (Leu) (D), are shown in spheres and CPK colouring. The coordinates were taken from PDB entry 1okc for BtAAC1 [9], from 2f2m for EmrE [55], from 1pv7 for LacY [54] and from 2a65 for LeuT [56].

direction, but the substrates can be exchanged in both directions in vitro. Therefore, either of the exchangeable substrates must be able to bind to the cytoplasmic state of the carrier as represented by the structure of the bovine ADP/ATP carrier [9]. The proposed binding site explains this property. Firstly, substrates that have similar functional groups and length may bind to the same site by small conformational changes in the substrate and interacting side chains. Secondly, the binding site has several different ways of accommodating substrates with similar chemical properties but of different length. For example, the Odc1p carrier can transport a variety of 2-oxo and 2-amino dicarboxylates (Fig. 7). H186 can interact with keto and amino groups (Fig. 7A and B) and for the second carboxylate there are different acceptors for different carbon chain lengths (K98, K94 and R95) (Fig. 7A, C and D). Thirdly, the part or moiety of the molecules that differs is not involved in binding. The ADP/ATP carrier should be able to bind all adenine nucleotides to the proposed binding site (Fig. 8). ADP and ATP are indeed able to bind, but in case of ATP one net negative charge remains as there are insufficient positively charged residues in the binding site. Thus, in the presence of a negative-inside membrane potential the movement of ATP in the direction of the mitochondrial matrix may be opposed. AMP is not transported by the ADP/ATP carrier [51], but in principle, it could bind to the site. However, binding of AMP does not involve residues at contact point III, which may be required as the trigger for translocation. It is also possible that K22 and R79 cannot bind the α-phosphate of AMP simultaneously, and so the substrate would carry a net negative charge and translocation against the negative-inside membrane potential would be opposed.

Transport of anions into mitochondria is opposed by the electrochemical gradient across the inner membrane. The basic residues of the binding site overcome the energetic barrier by compensating for the negative charges of a substrate. The citrate carrier, for instance, has one more positively charged residue in the binding site than the dicarboxylate carrier, Dic1p. The substrate NAD has two negative charges on the phosphates and one positive charge on the nicotinamide ring, producing a net negative charge of one. FAD has two phosphates and a net negative charge of two. In agreement, the NAD^+ carrier has one positively charged residue, whereas the FAD carrier has two positively charged residues in the substrate binding site.

Another way of driving anions into the mitochondrion against the electrochemical gradient is to couple their transport to cations, especially to Mg^{2+} or protons. For instance, Agc1p exchanges cytosolic glutamate plus a proton for aspartate from the mitochondrial matrix [52]. At physiological pH, E600 at contact point I would repel the carboxylate side chain of glutamate (Fig. 9A). However, if E600 is protonated, the binding to K604 could occur and transport could proceed with concomitant proton translocation. In other keto-acid carriers, such as Odc1p, Odc2p, Sfc1p and Ctp1p, the acidic and histidine residues in the vicinity of binding sites may fulfil a similar role. The binding site for the Mg^{2+}–ATP/Pi carrier Sal1p has a glutamate E318 in contact point I, which could be involved in the coordination of Mg^{2+} [53]. Without Mg^{2+} the substrate would be negatively charged and transport would be opposed by the membrane potential. In the case of the ADP/ATP carrier, the equivalent residue is T83 and it does not coordinate Mg^{2+}. The negative charges on ADP are neutralised by three positively charged residues in the binding site, explaining why ADP/ATP transport has no requirement for Mg^{2+} [51].

1.8. Position and threefold pseudo symmetry of the substrate binding site

Mitochondrial carriers have threefold pseudo symmetry [9]. The salt bridges formed between the signature motifs of the three repeats link H1, H3 and H5 together, closing the passage for the substrate (Fig. 1). The contact points of the substrate binding site are also related by threefold pseudo symmetry and are present on H2, H4 and H6. The salt bridges are all in one plane close to the border of the membrane on the matrix side, whereas the contact points for substrate binding are all in one plane at the midpoint of the membrane. The substrate is also at the midpoint of the membrane in four other families of transporters with different folds and mechanisms; the lactose permease LacY, a member of the major facilitator superfamily [54], the small multidrug transporter EmrE [55], a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters LeuT [56] (Fig. 10) and the glutamate transporters

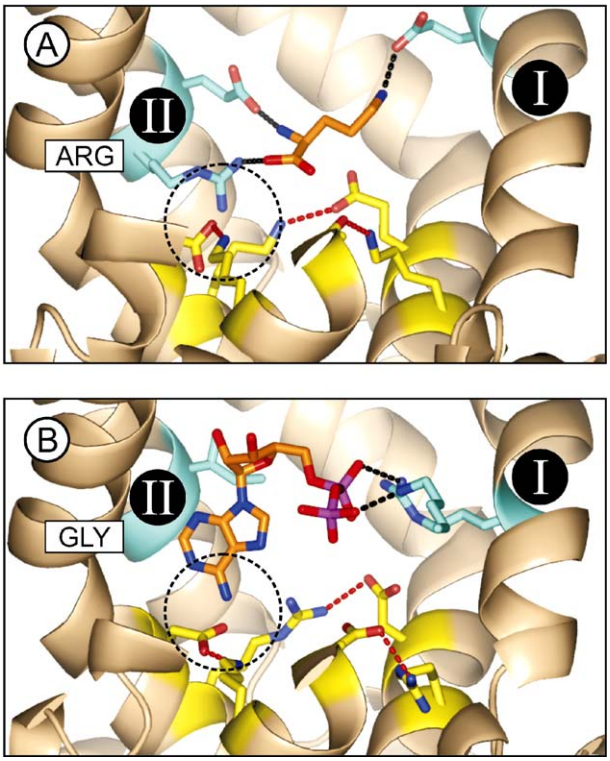

Fig. 11. The substrate binding site of the ornithine transporter Ort1p (A) and the ADP/ATP carrier BtAAC1 (B). Helices 1 and 6 and contact point III have been removed. The residues of the signature motif involved in the salt bridge network at the bottom of the cavity are depicted in yellow sticks. The residues of contact points I and II that are interacting with the substrate are shown in blue. The dotted circle indicates the close proximity of the NH1 or NH2 of the guanidino (A) and the N6 of adenine (B) to the negatively charged residue of the signature motif in the second repeat. The binding site of Ort1p is shown, but arginine is present in all amino acid and keto acid transporters, except ORNT2, which has a glutamine.

[57] (not shown). The water-filled cavities in these transporters allow the substrate to move to the midpoint in the membrane. During the transport cycle, transporters need to be closed on one or the other side of the membrane to prevent influx of protons and loss of the membrane potential. This is most easily achieved by the substrate being at the midpoint of the membrane, where it can function as a pivot point for structural changes around it.

1.9. Mechanism for substrate-induced carrier opening

The bound substrate is suspended between residues on α-helices H2, H4 and H6 at the bottom of a water-filled cavity, immediately above the salt bridges that close the carrier to the mitochondrial matrix (Fig. 11). The majority of the residues interacting with the substrate are charged and form salt bridges. Ionic interactions are stronger than hydrogen bonds and charged groups are less well shielded by the water molecules. The number of interactions between the substrate and the carrier are minimal and just enough to recognise the substrate, which may explain why the affinities for the substrates are in the micromolar range.

The location of the binding site suggests that substrate binding and carrier opening are coupled. This could be achieved by perturbation of the salt-bridge network through the introduction of a nitrogen atom close to the Asp or Glu of the signature motif of the second repeat. The nitrogen atom is either the N6 atom of the adenine moiety of adenine nucleotides or the guanidino NH1 or NH2 of arginine when it is bound to keto or amino acid substrates. Thus, the arginine functions as an adapter in two ways. First, the length of the arginine side chain ensures that the smaller substrates keto and amino acids can bind to the common binding site. In the adenine nucleotide transporters, the equivalent residue is glycine, which creates a pocket for the adenine ring. Second, the NH1 or NH2 of the guanidino group is in a similar position to the N6 nitrogen of the adenine ring, and both could trigger the rearrangement of the salt bridge network in a similar way. In the current state, the passage of the substrate to the matrix is prevented by inter-domain salt bridges formed by the charged residues of the signature motif from the three repeats (Stage 1 Fig. 12). The introduction of a nitrogen atom close to the negatively charged residue of the second repeat (Stage 2 Fig. 12) could lead to a rearrangement, in which salt bridges are formed between residues of the same signature motif (Stage 3 Fig. 12). When all three salt bridges have switched to intra-domain interactions, the α-helices at the bottom of the cavity are no longer bonded together and the passage to the

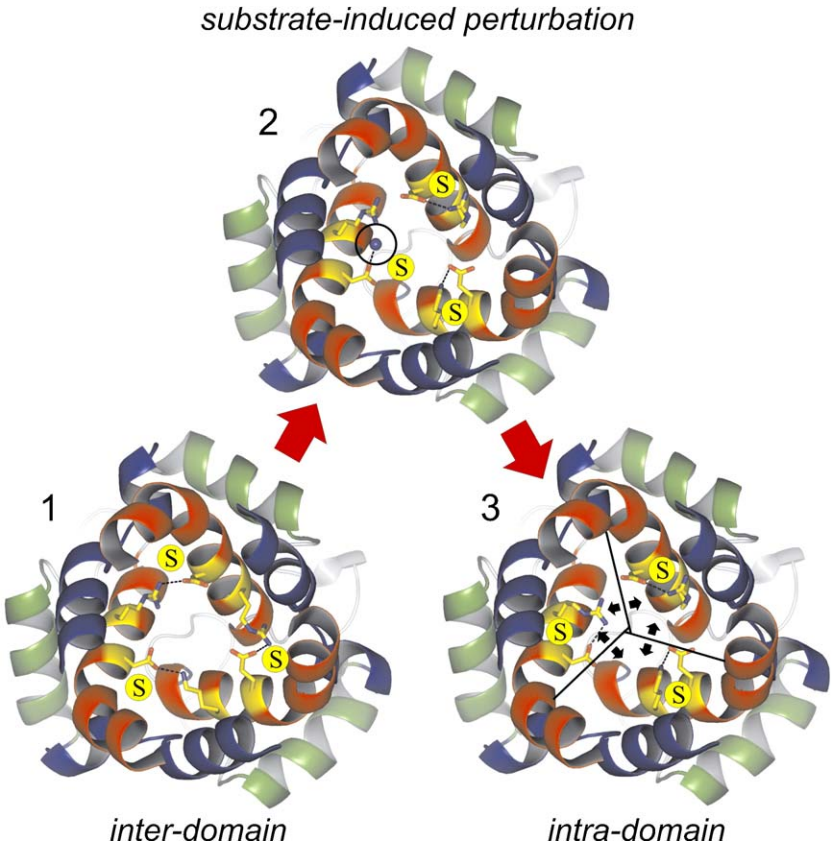

Fig. 12. Rearrangement of the salt bridge interactions between the signature motifs by substrate-induced perturbation. A view of the salt bridge network is shown in which the cytoplasmic half of the transporter has been removed. The salt bridges are yellow and are marked by the letter S. Panel 1 shows the state of the ADP/ATP carrier, in which the passage to the matrix is closed by inter-domain salt bridges formed by the signature motifs. Binding of the substrate (Panel 2) leads to the introduction of a nitrogen atom, either the N6 of adenine or NH1/NH2 of the guanidino group of arginine in position 182, close to the aspartate of the signature motif in the second repeat. A salt-bridge or hydrogen bond is formed between the substrate and Asp, leading to a rearrangement of the salt-bridges to all intra-domain interactions (Panel 3). In the final stage, the substrate is no longer bound to the signature motif and the carrier opens to the matrix side with concomitant movements of the transmembrane α-helices.

mitochondrial matrix is open. At the same time, substrate binding could pull α-helices H2, H4 and H6 closer together to allow a better fit of the substrate in the binding site, thereby providing energy for the structural changes with the substrate as the pivot point. Salt bridge rearrangement [10] and the participation of α-helix H4 in substrate translocation and conformational rearrangement [58,59] have been proposed previously as integral features of opening of mitochondrial carriers. The position and role of the substrate are consistent with the "single binding centre gated pore" model of the ADP/ATP carrier, in which a single binding site is alternatively accessible from the matrix and the cytosol [60], but a structure of the carrier in the matrix-state is required to confirm this.

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