Mitochondrial carriers in the cytoplasmic state have a common substrate binding site

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Mitochondrial carriers link biochemical pathways in the cytosol and mitochondrial matrix by transporting substrates across the inner mitochondrial membrane. Substrate recognition is specific for each carrier, but sequence similarities suggest the carriers have similar structures and mechanisms of substrate translocation. By considering conservation of amino acids, distance and chemical constraints, and by modeling family members on the known structure of the ADP/ATP translocase, we have identified a common substrate binding site. It explains substrate selectivity and proton coupling and provides a mechanistic link to carrier opening by substrate-induced perturbation of the salt bridges that seal the pathway to and from the mitochondrial matrix. It enables the substrate specificity of uncharacterized mitochondrial carriers to be predicted.

comparative modeling | membrane protein | sequence alignment

Members of the mitochondrial carrier family are located in the inner mitochondrial membrane and allow exchange of substrates between the cytosol and the mitochondrial matrix (1). The family is exclusive to eukaryotes, and its members are unrelated to other transporter families, including the major facilitator superfamily and the ABC transporters. Their substrates include nucleotides, amino acids, cofactors, carboxylic acids, and inorganic anions required by the organelle for oxidative phosphorylation, gluconeogenesis, synthesis and degradation of amino and fatty acids, macromolecular synthesis of proteins and nucleic acids, sterol metabolism, and thermogenesis (1). Mutations of the carriers are associated with diseases, including progressive external opthalmoplegia, adult- and neonatal-onset type II citrullinaemia, Amish-type microcephaly, hyperornithinemia-hyperammonia-homocitrullinuria, carnitine-acylcarnitine translocase deficiency, neonatal myoclonic epilepsy, severe obesity, and type II diabetes (1).

The mitochondrial carriers have three tandem repeats of ≈100 aa, each containing two transmembrane α-helices linked by a large loop (2) and a conserved signature motif P-X-[DE]-X-X-[RK] (3). The carriers exist in two distinct conformational states: the cytoplasmic state (*c*-state) in which the carrier accepts its substrate from the cytoplasm, and the matrix state in which it accepts a substrate from the mitochondrial matrix. For the ADP/ATP carrier, the inhibitor carboxyatractyloside (CATR) is known to bind to the carrier in the *c*-state only and prevent the binding of ADP. In the atomic model of the ADP/ATP carrier 1 from *Bos taurus* (BtAAC1) in complex with CATR (4), the six-transmembrane α-helices form a barrel that has pseudo-3-fold symmetry. CATR is bound in the internal aqueous cavity (see Fig. 4, which is published as supporting information on the PNAS web site), which is open to the cytosol and closed to the mitochondrial matrix, consistent with the structure representing the *c*-state. The prolines of the signature motif kink the transmembrane helices, bringing their C-terminal ends together at the base of the cavity. The charged residues of the signature motif lock these helices together with salt bridges and must be broken for a substrate to translocate to the mitochondrial matrix (4, 5). We have combined computational analysis based on the BtAAC1 structure with experimental evidence to deduce the position of a common substrate binding site in the *c*-state of mitochondrial carriers from *Saccharomyces cerevisiae*.

Results and Discussion

Classification of Mitochondrial Carriers. The mitochondrial carriers of *S. cerevisiae* were chosen for this study, as more have been identified than in any other species. Nineteen carriers have been characterized by transport assays on purified reconstituted proteins and substrates for five more carriers have been proposed from genetic studies (see Table 1 and Table 2, which is published as supporting information on the PNAS web site). To avoid using incorrectly attributed substrates, we considered only the carriers identified by transport assays.

The carriers were classified into three major subfamilies on the basis of similarities in their substrate functional groups (see Table 2 and Table 3, which is published as supporting information on the PNAS web site): keto acid carriers (Ctp1p, Dic1p, Oac1p, Odc1/2p, and Sfc1p), amino acid carriers (Agc1p, Crc1p, Ort1p, and Pet8p) and carriers of adenine-containing substrates (Aac1p, Aac2p, Aac3p, Ant1p, Pet8p, and Sal1p).

Comparative Modeling of the Yeast Mitochondrial Carriers. In the sequence alignment of mitochondrial carriers from many species, conserved residues were found mainly in transmembrane helices rather than in extramembranous loop regions (see Fig. 5, which is published as supporting information on the PNAS web site). The residues facing the lipid bilayer are hydrophobic, and most residues facing the inner cavity are charged and polar. The latter residues may participate in substrate binding, the translocation mechanism, or structural integrity of the carrier. But on the basis of conservation their role cannot be established. The overall sequence similarity among the members of the family allowed comparative models of the yeast carriers to be built based on the structure of BtAAC1 (4), but there are variations in the signature motif P-X-[DE]-X-X-[KR]. First, Flx1p, Rim2p, YEL006w, and YIL006w have tryptophan at 134_{BtAAC1} instead of the expected acidic residue, but they have retained the second component of the salt bridge, the basic residue at 234_{BtAAC1}. This interaction was modeled as a cation–π interaction (6). Second, proline is replaced by serine in YMR166c (S73), Aac1p (S138), Aac2p (S147), and Aac3p (S136), and so its $C_\beta O$ was hydrogen-bonded to the main chain to mimic a proline.

The Binding Site of Keto Acid and Amino Acid Carriers. The amino acid and keto acid substrates of the mitochondrial carriers are substituted carboxylic acids with a variable group (see Fig. 6, which is published as supporting information on the PNAS web site). The carriers transport structurally related substrates, sug-

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Abbreviations: *c*-state, cytoplasmic state; CATR, carboxyatractyloside; BtAAC1, ADP/ATP carrier 1 from *Bos taurus*; ORNT, ornithine transporter.

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Table 1. Mitochondrial carriers of yeast (Sc) plus selected human (Hs) and bovine (Bt) orthologs

		Group		Contact point		
Carrier	Substrate	R	K	I	II	III
BtAAC1	ADP	Phosphate	Adenine	R-T-N	G-I	R
ScAac1/2/3p	ADP	Phosphate	Adenine	R-T-N	G-I	R
ScSal1p	MgATP	Phosphate/Mg^{2+}	Adenine	K-E-K	G-I	K
ScLeu5p	CoA*	Phosphate/amide	Adenine	R-Y-K	G-M	K
ScFlx1p	FAD*	Flavin	Adenine	G-A-Y	G-V	R
ScRim2p	Unknown	—	—	G-A-N	G-S	R
ScYPR011c	Unknown	—	—	R-Y-Q	G-V	K
ScYEL006w	Unknown	—	—	G-T-Y	G-M	R
ScYIL006w	Pyruvate*	Methyl	Keto acid	G-T-Y	G-L	R
HsMFTC	Folate*	ND	ND	G-S-Y	G-T	R
ScTpc1	ThPP	Phosphate	Thiamine	L-Y-Q	T-I	K
ScAnt1p	ATP	Phosphate	Adenine	A-Q-Y	L-T	K
ScGgc1p	GTP	Phosphate	Guanine	Y-Q-K	R-N	T
ScOac1p	Oxaloacetate	Carboxylate	Keto acid	Y-L-R	R-T	R
ScDic1p	Malate	Carboxylate	Hydroxy acid	R-Y-R	R-G	R
ScOdc1p	2-Oxoglutarate	Carboxylate	Keto acid	M-K-K	R-H	R
ScOdc2p	2-Oxoglutarate	Carboxylate	Keto acid	M-K-K	R-N	R
ScSfc1p	Succinate	Carboxylate	Carboxylate	G-K-R	R-Q	R
ScCtp1p	Citrate/malate	Carboxylate	Hydroxy acid	G-K-R	R-Q	R
ScYhm2p	Unknown	—	—	I-T-V	R-Q	L
ScYmc2p	Unknown	—	—	G-C-Q	R-A	R
ScYFR045w	Unknown	—	—	A-K-R	R-Q	K
HsOGC	2-Oxoglutarate	Carboxylate	Keto acid	R-Y-R	R-A	R
HsUCP1	Fatty acid/H^{+}*	ND	ND	R-S-R	R-S	R
ScPic2p	Phosphate	ND	ND	G-Q-K	R-Q	M
ScMir1p	Phosphate	ND	ND	G-Q-K	K-Q	M
ScAgc1p	Glutamate	Carboxylate	Amino acid	G-E†-K	R-D	R
ScCrc1p	Acetylcarnitine	Methyl ester	Amino acid	G-I-S	R-D	R
ScPet8p	SAM	Adenine	Amino acid	A-G-F	R-E	W
ScYmc1p	Unknown	—	—	G-C-Q	R-E	R
ScYDL119c	Unknown	—	—	R-G-Y	R-D	R
ScYMR166c	Unknown	—	—	G-S-F	R-D	W
ScMtm1p	Mn cofactor*	ND	ND	M-A-Y	R-D	K
ScOrt1p	Ornithine	Amine	Amino acid	G-E-L	R-E	R
HsORNT1	Ornithine	Amine	Amino acid	A-E-L	R-E	R
HsORNT2	Ornithine	Amine	Amino acid	A-E-L	Q-E	R
ScMrs3/4p	Iron*	ND	ND	G-A-Y	M-N	A

The groups R and K of the substrate interact with residues at contact point I ($79/83/87_{BtAAC1}$) and II ($182–183_{BtAAC1}$), respectively, of the proposed binding site. Residues that interact with the substrate in modeled structures are underlined. ND, interacting substrate moiety is not determined.

*Substrate not confirmed by transport assay.

†E600 could form a proton-mediated carboxyl-carboxylate interaction with the substrate.

gesting the existence of a common binding site and mechanism of carrier opening. A systematic search of the cavity of the model of each carrier identified residues that satisfied the constraints for binding of their substrates. Fifty-six potential binding sites were common to all carriers (see Table 4, which is published as supporting information on the PNAS web site), but only six distinct contact points were predicted to bind the keto and amino groups of the substrates: $182/183_{BtAAC1}$, $182/235_{BtAAC1}$, $32/235_{BtAAC1}$, $137/235_{BtAAC1}$, $190/183_{BtAAC1}$, and $279/235_{BtAAC1}$. A binding site must discriminate between the amino and keto groups, and contact points with a polar residue at 235_{BtAAC1} do not satisfy this criterion and were rejected. The contact point $190/183_{BtAAC1}$ was rejected because steric interference from the intervening residues $186–187_{BtAAC1}$ would prevent the simultaneous binding of the carboxylate and the amine or keto groups. This left $182/183_{BtAAC1}$ as the only remaining solution (Table 1). The residues at this contact point can recognize and discriminate amino and keto acids, because the amino acid carriers have the motif R-[DE] that is complementary to the zwitterionic amino acid, whereas the keto acid carriers have the basic motif R-[QHNT], favoring the electronegative keto acid.

Next, the contact point for the variable group of the substrate was considered. With the amino or keto acid bound to $182–183_{BtAAC1}$, possible contact points for the substrate variable group were shared by all carriers in the neighborhoods of 83_{BtAAC1}, 118_{BtAAC1}, and 194_{BtAAC1} (Table 4). The polar residues around 118_{BtAAC1} and 194_{BtAAC1} were rejected as they cannot discriminate between acidic and basic groups in substrates. The residues in the vicinity of 83_{BtAAC1} remained as the only plausible solution. In all of the amino acid and keto acid carriers, residues at this point can differentiate the variable group of the substrates (Table 1). When the substrate has a basic moiety complementary acidic residues are present (e.g., E83 in Ort1p); when they have acidic functional groups basic amino acids are present (e.g., K601 and K604 in Agc1p); substrates with hydrophobic groups have a matching hydrophobic surface (e.g., I89 and F90 in Crc1p), and aromatic groups in substrates make stacking interactions with aromatic residues (e.g., F68 in Pet8p). The vicinity of 83_{BtAAC1}

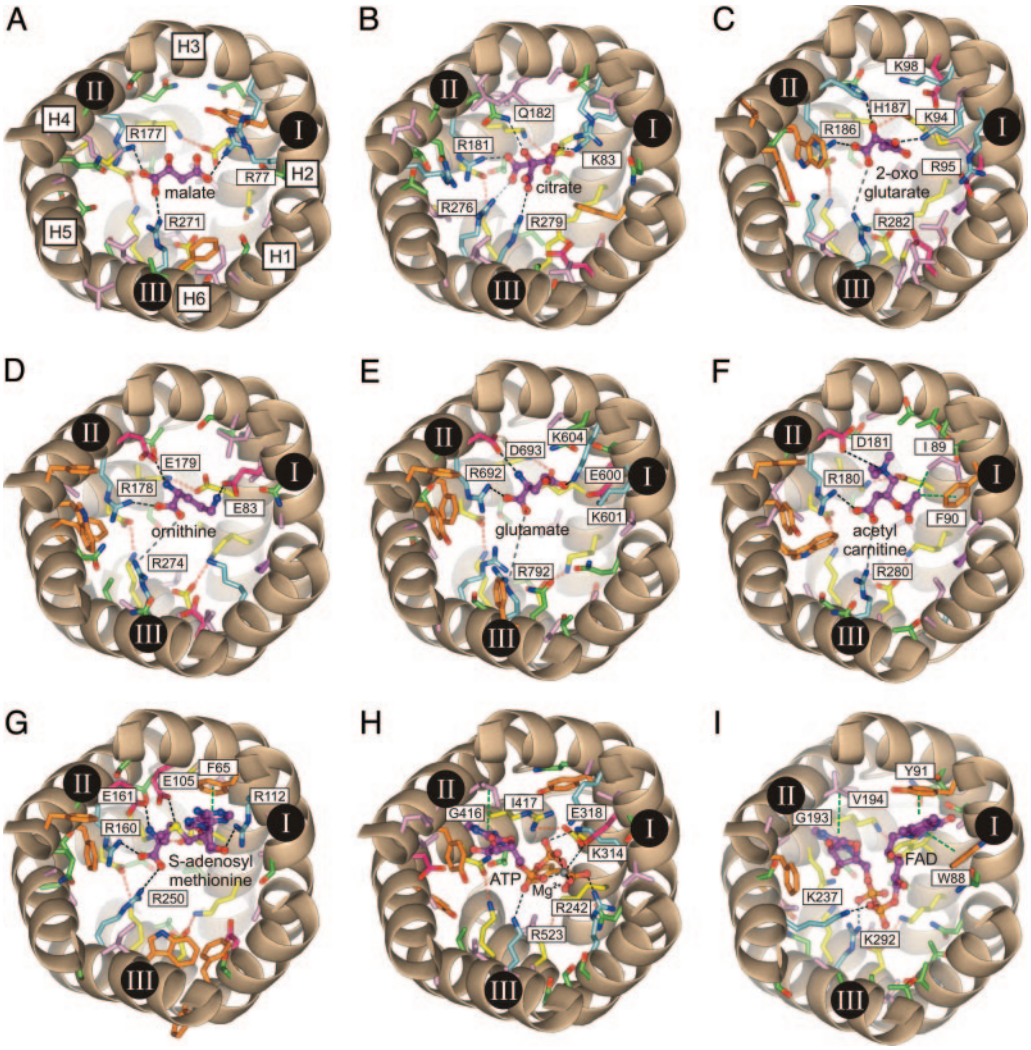

Fig. 1. Models of the yeast carriers viewed from the mitochondrial intermembrane space. Substrates (purple) are bound to contact points I, II, and III. (*A*) Dic1p. (*B*) Ctp1p. (*C*) Odc1p. (*D*) Ort1p. (*E*) Agc1p. (*F*) Crc1p. (*G*) Pet8p. (*H*) Sal1p. (*I*) Flx1p. Salt bridge residues (yellow), plus basic (cyan), acidic (magenta), other polar (green), aromatic (orange), and aliphatic (pink) residues are shown. Salt bridge interactions at the base of the cavity are indicated by red dashed lines. Interactions between the substrate and carrier are indicated: electrostatic/polar (black dashed line), hydrophobic (green dashed line), and via bridging water (gray dashed line).

and the region of $182–183_{BtAAC1}$ are defined as contact points I and II. Models of the amino acid and keto acid carriers with the docked substrates are shown in Fig. 1.

The Binding Site of Adenine Nucleotide Carriers. The substrates of the adenine nucleotide carriers have greater conformational flexibility and more functional groups than those of the amino acid and keto acid carriers. In many proteins, the adenine moiety of ADP is in a hydrophobic pocket and the phosphates interact with basic residues (7). A superposition of ADP and the diterpenoid moiety of CATR shows their structural similarity (see Fig. 7, which is published as supporting information on the PNAS web site). The glycosidic diterpenoid CATR was replaced by ADP in the atomic model of BtAAC1 (Fig. 2). The adenine ring replaces the diterpenoid's bicyclo-*cis*-decalin ring in the hydrophobic pocket formed by G182, I183, and Y186, N1 is in the position of the diterpenoid ethylene group, and N6 is in the position of the diterpenoid's hydroxyl group, placing it close to the salt bridges. The ribose of ADP replaces the cyclohexane ring of CATR, and its α- and β-phosphates replace the carboxylates of CATR and interact with K22, R79, and R279. Thus, the diterpene moiety of CATR mimics ADP, and the interactions of the sulfate groups of the glycosidic moiety likely prevent progression to the matrix state. Four pieces of experimental evidence support ADP binding to this site. First, binding of CATR prevents the binding of ADP in the *c*-state (8). Second, mutation of residues corresponding to K22, R79, Y186, and R279 abolishes transport activity of the yeast ADP/ATP carrier (4, 9, 10). Third, the photoaffinity analogue 2-azido[α-^{32}P]adenosine diphosphate labels I183 of BtAAC1 preferentially (11). Fourth, the mutation of the equivalent $G182_{BtAAC1}$ in the proposed mitochondrial folate transporter/carrier (12) has a detrimental phenotypic effect.

Thus, the ADP binding site is formed by K22, R79 (contact point I), G182-I183 (contact point II), and R279 (defined as contact point III), demonstrating the common binding site in adenine, amino acid, and keto acid carriers (Table 1).

The binding site explains why ADP and ATP are bound and transported by the ADP/ATP carrier, whereas AMP, GDP, and GTP are not (13). Although AMP could bind to the site, it lacks the interaction with the residue R279 on helix H6 (contact point III). GDP and GTP are not bound and transported, because the

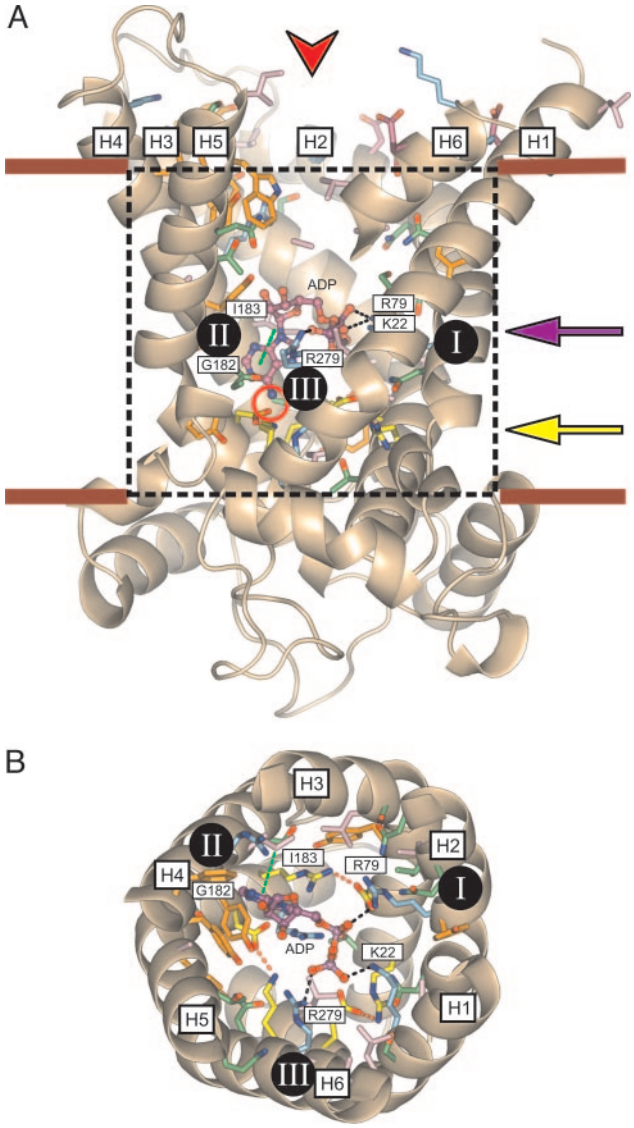

Fig. 2. Model of ADP (purple) bound to contact points I, II, and III of BtAAC1. (*A*) Lateral view with the residues 276–285 of H6 removed. The red circle highlights the proximity of the adenine's N6 to a salt bridge. The transmembrane sector is outlined, and the position of salt bridges (yellow arrow) and substrate (purple arrow) is shown. (*B*) View of the transmembrane sector from the mitochondrial intermembrane space. Coloring scheme is the same as in Fig. 1.

N6 hydrogen bond donor in adenine is replaced by a hydrogen bond acceptor oxygen in guanine and the polar amino group on C2 of guanine would be directed into the hydrophobic pocket.

The binding of MgATP to Sal1p was modeled in a similar manner as ADP to BtAAC1 (Fig. 1) with the phosphate groups interacting with R242 (22_{BtAAC1}) and K314 (79_{BtAAC1}) at contact point I and the adenine moiety in a hydrophobic pocket formed by G416 and I417 ($182–183_{BtAAC1}$) at contact point II. At contact point I of Sal1p, the phosphate-coordinated magnesium cation interacts with E318 (83_{BtAAC1}), whereas in BtAAC1 a threonine is in the equivalent position, which explains why Sal1p transports MgATP rather than ADP (14). Similarly, in Flx1p the flavin ring forms an aromatic stacking interaction with W88 (84_{BtAAC1}) and Y91 (87_{BtAAC1}) at contact point I, the adenine ring of FAD occupies the hydrophobic pocket formed by G193 and V194 ($182–183_{BtAAC1}$) at contact point II and its β-phosphate interacts with K237 (220_{BtAAC1}) (Fig. 1).

The GDP/GTP and Phosphate Carriers. The Ggc1p carrier transports the nucleotides GDP and GTP. The R-N motif at its contact point II suggests that Ggc1p could have evolved from a keto acid carrier. Thus, the binding of GDP may mimic the binding mode of a keto acid in which either the oxygen atom on C6 of the guanine ring or the phosphate groups interacts with $R182_{BtAAC1}$ at contact point II. That the binding site is not similar to that of the carriers of adenine-based substrates is unsurprising given the different chemical properties of the two nucleotides.

The phosphate carrier Mir1p has basic residues at contact points I and II that mutagenesis shows are important for its transport function (15). The binding site is similar to that of keto acid carriers, but a single phosphate anion is too small to be bound at contact points I and II simultaneously. It is possible that Mir1p could either bind two phosphates or it functions as a phosphate-keto acid exchanger, like Dic1p.

The Binding Site of the Mitochondrial Carriers. In the aligned sequences of the characterized yeast mitochondrial carriers (see Fig. 8, which is published as supporting information on the PNAS web site), many amino acids in the transmembrane helices are conserved in all carriers. The amino acids conserved at a number of positions correlate with the subfamily of the carriers. The most striking correlation is at contact point II (Table 1 and Fig. 9, which is published as supporting information on the PNAS web site), where all of the amino acid carriers possess the motif R-[DE], all keto acid carriers have R-[QHNT], and all of the adenine nucleotide carriers have G-[IVLM]. Thus, residues of contact point II recognize and distinguish between amino acids, keto acids, and nucleotides, and those at contact point I interact with the variable group of the substrates and confer specificity. It is apparent that the binding site of the amino acid and keto acid carriers is smaller than that of the adenine nucleotide carriers, in which the small, pocket-forming $G182_{BtAAC1}$ that accommodates the adenine moiety is replaced by $R182_{BtAAC1}$ with its large side chain.

Mutagenesis studies have shown that the residues of contact point I are important for function in yeast Aac2p (9) and Mir1p (15) and human UCP1 (16) and carnitine-acylcarnitine translocase (17). Mutation of residue 182_{BtAAC1} at contact point II affect carrier function in yeast Mir1p (15) and Ctp1p (18), human OGC (19) and UCP1 (20), and hamster mitochondrial folate transporter/carrier (12). Hyperornithinemia-hyperammonia-homocitrullinuria syndrome is associated with the mutation E180K in ornithine transporter (ORNT) 1 (21). Although the mutant protein is stable and properly targeted, it cannot transport basic amino acids. The mutation is at 183_{BtAAC1} of contact point II and it changes the motif from R-E to R-K, which would disfavor ornithine from binding. An isoform of the ORNT (ORNT2) cannot replace fully the function of ORNT1 (22). It has the motif Q-E at contact point II, and so the amino acid group of ornithine would bind less well than to wild-type ORNT1, consistent with its reduced transport capability.

Residue 279_{BtAAC1} of contact point III is a well conserved arginine throughout the family (Table 1 and Fig. 8). In BtAAC1, R279 interacts with CATR via a bridging water molecule. In the structural models, this residue could interact with anionic functional groups of the substrate, either directly or via a bridging water molecule (Fig. 1). Mutagenesis of residue 279_{BtAAC1} (contact point III) affects carrier activity in Aac2p (10), and in the human ORNT1 is associated with hyperornithineamia-hyperammonia-homocitrullinuria syndrome (23). Although it does not confer substrate specificity, it could constitute a third important contact point of the substrate-binding site. In fact, residues 79_{BtAAC1} (contact point I), 182_{BtAAC1} (contact point II), and 279_{BtAAC1} (contact point III) are equivalent in position because of the pseudo-3-fold symmetry of the carriers.

Many carriers can transport a range of structurally related

substrates, which can be achieved in three ways. First, the residue of a contact point can accept groups with similar chemistry. For example, E83 of contact point I in Ort1p can bind the basic groups of ornithine, arginine, and citrulline. Second, variability in the length of the substrate is tolerated by using alternative residues to bind the variable group. For example, Odc1p has three basic residues at contact point I and transports the related substrates 2-oxoadipate, 2-oxoglutarate, and 2-oxopimelate. Multiple basic residues at contact point I are also present in Dic1p, Sfc1p, and Ctp1p. Third, the distinguishing functional groups of similar substrates are not bound, for example, the γ-phosphate of ATP in the ADP/ATP carriers.

Anion transport 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 the negative charges of a substrate. For example, during import of ADP by the ADP/ATP carrier, the phosphate groups are neutralized by interaction with K22, R79, and R279. Because the charge on the γ-phosphate of ATP is not compensated, its import is disfavored. Another way of driving anions into the mitochondrion against the electrochemical gradient is to couple their transport to cations. For instance, Agc1p exchanges cytosolic glutamate plus a proton for aspartate from the mitochondrial matrix (24). At physiological pH, E600 (83_{BtAAC1}) could repel the carboxylate side chain of the glutamate substrate bound to K604 (87_{BtAAC1}) at contact point I (Fig. 1). However, if E600 is protonated by K601 or K604, the glutamate substrate can interact with E600, with the proton coupling mediated as a carboxyl-carboxylate interaction between substrate and carrier (25). E600 is shielded from the approach and binding of basic substrates from the cytosol by K601 and K604. In other keto acid carriers, for example, Odc1/2p, Sfc1p, and Ctp1p, the acidic and histidine residues in the vicinity of the binding site may fulfill the same role.

Coupling of Substrate Binding to Carrier Opening. The substrate binding site is at the midpoint of the membrane, as it is also in lactose permease (26) and a bacterial homologue of the Na^+/Cl^--dependent neurotransmitter transporters (27), although there is no structural similarity between these transporters. The bound substrate is suspended between residues on helices H2, H4, and H6 at the bottom of a water-filled cavity, immediately above the salt bridges that seal the carrier to the mitochondrial matrix (Fig. 2). Substrate binding induces a series of events that culminates in the carrier opening to the mitochondrial matrix. We propose that the binding of a substrate to H2, H4, and H6 allows H1, H3, and H5 to rotate or move, and thus the bound substrate provides the fulcrum about which the transmembrane helices pivot as the carrier opens to the mitochondrial matrix. The location of the binding site suggests that substrate binding and carrier opening are coupled by the introduction of charged groups perturbing the salt bridges, causing them to rearrange to a configuration permitting substrate translocation. Candidates are either the N6 of adenine or the guanidino of $R182_{BtAAC1}$ when it is bound to keto acid and amino acid substrates. Substrate binding could pull helices H2, H4, and H6 closer together to allow a better fit of the substrate in the binding site, thereby providing energy for subsequent events, including the conversion from the *c*-state to the matrix state (28). It is possible that this site is consistent with the "single binding center gated pore" model of the ADP/ATP carrier, in which a single binding site is alternatively accessible from the matrix and the cytosol (29), but we cannot confirm this in the absence of a structure for the matrix state. Salt bridge rearrangement (5) and the participation of helix H4 in substrate translocation and conformational rearrangement (30, 31) have been proposed previously as integral features of carrier opening.

Substrate Specificity of Uncharacterized Carriers. The substrate specificity of mitochondrial carriers based on genetics studies was examined in light of the residues at the proposed binding site (Table 1 and Fig. 9). Rather than having the features of a keto acid carrier, the proposed pyruvate carrier (32), YIL006w, has a binding motif typical of an adenine nucleotide carrier. Leu5p also has a contact point II predicting adenine nucleotide binding, which is consistent with it being the coenzyme A carrier (33). Mtm1p, which is proposed to transport a manganese cofactor (34), has a contact point II typical of an amino acid carrier, and the proposed iron transporters Mrs3p and Mrs4p (35) share a novel M-N motif at contact point II, suggesting that they transport neither nucleotides nor keto acids or amino acids and could form a distinct subfamily transporting coordinated iron. The residues at contact point II of Rim2p, YEL006w, and YPR011c are typical of adenine nucleotide carriers, those of YDL119c, Ymc1p, and YMR166c are characteristic of amino acid carriers, and that of Ymc2p, which is homologous to Ymc1p, is similar to that of a keto acid carrier. As Ymc1p and Ymc2p have identical residues at contact point II, they probably transport substrates differing only in their substituted carboxylic acid, such as those coupled by a mitochondrial aminotransferase.

The residues forming the common binding site in yeast mitochondrial carriers in the *c*-state are conserved in human orthologs. Therefore, the human carriers likely operate by a similar mechanism and the substrate specificities of uncharacterized human mitochondrial carriers can be examined in a similar way.

The Uncoupling Protein UCP1. The human uncoupling protein UCP1 is widely believed to transport protons, although fatty acids have been proposed as substrates (20). It is inhibited by ADP and GDP (20), and mutations of $R83I_{(79_{BtAAC1})}$, $R91T_{(87_{BtAAC1})}$, $R182Q_{(182_{BtAAC1})}$, and $R276Q_{(279_{BtAAC1})}$ decrease or abolish nucleotide binding (16, 20). These residues are similar to residues at contact points I, II, and III of the yeast keto acid carriers and the GDP/GTP-binding Ggc1p (Table 1 and Fig. 9), suggesting that UCP1 shares the common binding site of the other carriers, that the physiological substrate has carboxylate groups and the inhibitory nucleotides compete for the same site.

Methods

Comparative Modeling. Protein sequences of the yeast mitochondrial carriers were taken from the *Saccharomyces* Genome

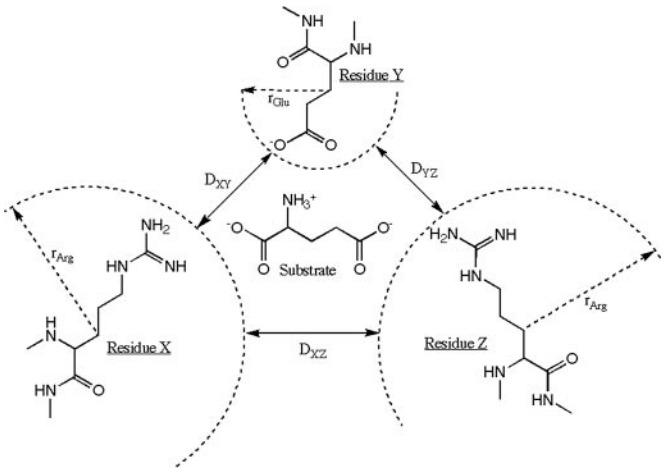

Fig. 3. Determination of substrate binding sites in the structures of modeled mitochondrial carriers. The residues X, Y, and Z each interact with a single functional group of the substrate. $r_{\alpha\alpha}$, maximum reach of an amino acid side chain. D_{ij}, separation between a pair of amino acids used in the distance constraint test.

Database (36). Orthologs of the yeast proteins were aligned by using CLUSTALW (37) with the secondary structure of BtAAC1 used to weight gap penalties. To facilitate comparison among the mitochondrial carriers, BtAAC1 is used to define a common residue numbering system. For example, K179 of Mir1p aligns unambiguously with G182 of BtAAC1 and has a relative position of 182_{BtAAC1}.

The pairwise alignment of each yeast protein with BtAAC1 was taken from the multiple sequence alignment, and MODELLER (38) was used to calculate a comparative model of the yeast protein. MOLPROBITY (39) was used to check the ψ, φ, and C_β angles of residues of the models.

Identification of a Common, Substrate-Specific Binding Site. The functional groups of a substrate define its physiochemical properties. Substrate recognition by proteins requires complementary interactions between its amino acids and the functional groups of the substrate. For each substrate, the chemical properties of the functional groups were identified plus the distance between each pair of functional groups (see Tables 5 and 6, which are published as supporting information on the PNAS web site). These features impose constraints on residues that constitute a binding site, thus the cavity of each modeled carrier was searched systematically for sets of residues that satisfied the required distance and chemical constraints to bind a substrate, as defined in Fig. 3. A set of residues was rejected as a plausible binding site if either the substrate–residue interaction was unfavorable or the residues were too far apart to bind the substrate. The binding sites of the carriers were compared to determine whether any occurred in equivalent positions for all carriers and thus may constitute a common binding site across members of the family (see Fig. 10, which is published as supporting information on the PNAS web site). Finally, PYMOL was used to dock ligands into the discovered binding sites.

For additional details of methods, see *Supporting Methods*, which is published as supporting information on the PNAS web site.

Note Added in Proof. The previously proposed pyruvate carrier, YIL006w, has now been identified and functionally characterized as the mitochondrial NAD carrier protein (Ndt1p) by using transport assays with purified protein reconstituted into liposomes (40). Compared with the pyruvate substrate, the adenine-containing NAD substrate is consistent with the "G-L" motif found at contact point II and aromatic stacking at contact point I.

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