CHAPTER EIGHT

Antiporters of the Mitochondrial Carrier Family

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Abstract

The eukaryotic transport protein family SLC25 consists of mitochondrial carriers (MCs) that are recognized on the sequence level by a threefold repeated and conserved signature motif. The majority of MCs characterized so far catalyzes strict exchanges of substrates across the mitochondrial inner membrane. The substrates are nucleotides, metabolic intermediates, and cofactors that are required in cytoplasmic and matrix metabolism. This review summarizes and discusses the current knowledge of the antiport mechanism(s) of MCs that has been deduced from determining transport characteristics and by analyzing structural, sequence, and mutagenesis data. The mode of transport varies among different MCs with respect to how the substrate translocation depends on the electrical and pH gradients across the mitochondrial inner membrane,

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for example, the ADP/ATP carrier is electrogenic (electrophoretic), the GTP/GDP carrier is dependent on the pH gradient, the aspartate/glutamate carrier is dependent on both, and the oxoglutarate/malate carrier is independent of them. The structure of the bovine ADP/ATP carrier consists of a six-transmembrane α-helix bundle with a pseudo-threefold symmetry and a closed matrix gate. By using this structure as a template in homology modeling, residues engaged in substrate binding and the formation of a cytoplasmic gate in MCs have been proposed. The functional importance of the residues of the binding site, the matrix, and the cytoplasmic gates is supported by transport activities of different MCs with single point mutations. Cumulative evidence has been used to postulate a general transport mechanism for MCs.

ABBREVIATIONS

AAC ADP/ATP carrier
AGC aspartate/glutamate carrier
APC ATP-Mg^{2+}/P_i carrier
CAC carnitine/acylcarnitine carrier
CIC citrate carrier
DTC dicarboxylate–tricarboxylate carrier
GC glutamate carrier
Ggc GTP/GDP carrier
MC mitochondrial carrier
MCF mitochondrial carrier family
OGC oxoglutarate carrier
P_i inorganic phosphate
PiC phosphate carrier

1. INTRODUCTION

Mitochondrial carriers (MCs) transport nucleotides, amino acids, carboxylic acids, inorganic ions, and cofactors across the mitochondrial inner membrane, thereby connecting metabolic pathways of the cytoplasm with those of the mitochondrial matrix. For example, MCs provide substrates for oxidative phosphorylation, such as ADP and phosphate (P_i) transported by the ADP/ATP carrier (AAC) and phosphate carrier (PiC), respectively. MCs also transport reducing equivalents of NADH for respiratory complex I by the malate/aspartate shuttle, which employs the following two membrane transporters: the aspartate/glutamate carrier (AGC) and the oxoglutarate carrier (OGC). Moreover, transport steps catalyzed by MCs play roles in gluconeogenesis, thermogenesis, fatty acid and amino acid metabolism, as well as for mitochondrial replication, transcription, and

translation. The importance of MCs in intermediary metabolism is illustrated by the fact that severe diseases are caused by mutations in the genes of certain MCs (Lindhurst et al., 2006; Palmieri, 2008, 2013).

MCs are encoded by the nuclear genes of the SLC25 family (Palmieri, 2013), translated in the cytosol, targeted to mitochondria with the help of chaperones (Zara, Ferramosca, Palmisano, Palmieri, & Rassow, 2003; Zara, Ferramosca, Robitaille-Foucher, Palmieri, & Young, 2009; Zara, Palmieri, Mahlke, & Pfanner, 1992), and inserted into the inner membrane with the N- and C-termini in the intermembrane space (Bisaccia, Capobianco, Brandolin, & Palmieri, 1994; Capobianco, Bisaccia, Michel, Sluse, & Palmieri, 1995; Capobianco, Brandolin, & Palmieri, 1991; Palmieri, Bisaccia et al., 1993) by a specific insertion machinery (Endres, Neupert, & Brunner, 1999). Although the majority of the mitochondrial carrier protein family (MCF) members are found in mitochondria, a few of them have been localized to peroxisomes and chloroplasts (Agrimi, Russo, Pierri, & Palmieri, 2012; Agrimi, Russo, Scarcia, & Palmieri, 2012; Palmieri et al., 2009; Palmieri, Rottensteiner et al., 2001).

MCs are characterized by a tripartite structure consisting of three tandemly repeated domains of about 100 amino acids (Saraste & Walker, 1982). Each domain forms two hydrophobic transmembrane segments with connecting loops, and contains a signature sequence motif PX[D/E]XX[K/R]X[K/R] (20–30 residues) [D/E]GXXXX[W/Y/F][K/R]G (PROSITE PS50920, PFAM PF00153, and IPR00193) (Palmieri, 1994, 2004). This motif has been used to identify MCs in eukaryotic genomic sequences; 53 MCF members are found in man, 35 in yeast, and 58 in *Arabidopsis thaliana* (Palmieri, Palmieri, Runswick, & Walker, 1996; Palmieri & Pierri, 2010a; Picault, Hodges, Palmieri, & Palmieri, 2004). About half of these have been functionally characterized, that is, their substrates and possible physiological roles have been identified by direct transport assays (Palmieri, 2004, 2013; Palmieri, Agrimi et al., 2006; Palmieri, Runswick, Fiermonte, Walker, & Palmieri, 2000). Currently, MCs can be divided into four major classes or groups depending on the type of substrates they transport (nucleotides/dinucleotides, carboxylic acids, amino acids, and other substrates) and into subfamilies containing all homologues that transport the same substrate (Palmieri, Pierri, De Grassi, Nunes-Nesi, & Fernie, 2011).

Only the atomic structures of the carboxyatractyloside-inhibited bovine AAC1 and yeast AAC2 and AAC3 have been determined by X-ray crystallography (Pebay-Peyroula et al., 2003: Ruprecht et al., 2014).

These structures have pseudo-threefold symmetry and consist of six-transmembrane α-helices (H1-H6) forming a basket-like structure (Fig. 8.1) closed toward the matrix side by a salt-bridge network formed by the two conserved charged residues that follow the proline in the signature motif. The salt-bridge network is accomplished by the close proximity of the lower part of the odd-numbered α-helices owing to the kink of the α-helices at the level of the prolines of the motifs. On the matrix side, the odd- and even-numbered transmembrane α-helices are connected by small α-helices (h_{12}, h_{34}, h_{56}) that are terminated by the first glycine in the second part of the signature motif. All MCs are thought to have the same basic fold as represented by the structure of bovine AAC1.

Many of the MCF members have been shown experimentally to be antiporters *in vitro* (Table 8.1) (Krämer & Palmieri, 1989; Palmieri, 2013; Palmieri, Lasorsa et al., 2000). When reconstituted into liposomes, these MCs do not transport externally added radioactive substrate unless a counter substrate is loaded inside the proteoliposome. The substrate gradient with a high concentration of cold substrate internally drives the exchange. However, although MCs are considered antiporters based on *in vitro* transport

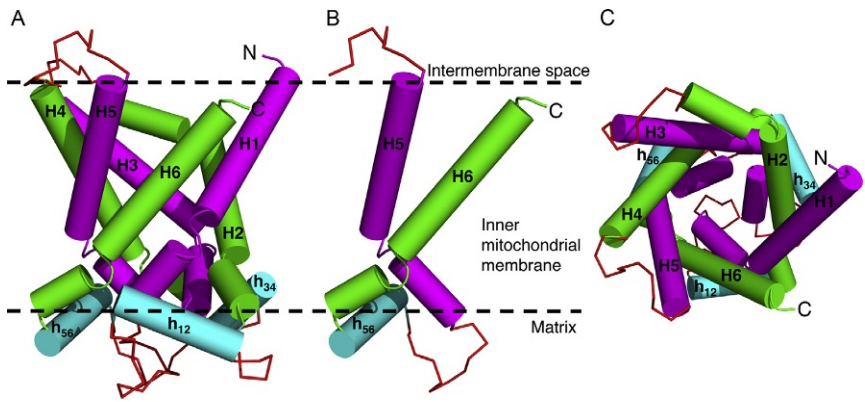

Figure 8.1 Structure of the bovine AAC1. The odd- and even-numbered α-helices are colored in magenta and green, respectively; loops are in red and the matrix α-helices are in cyan. The signature motif of the MCF is shown at the bottom of the figure. (A) Viewed from the lateral membrane side. (B) The last 100-residue repeat. The kinks in the odd-numbered α-helices are induced by the proline in the signature motif. The even-numbered α-helices initiate with the first glycine in the signature motif, and the kinks are induced by the second glycine. (C) View into the central cavity from the intermembrane space side.

Table 8.1 MCF members that are typical antiporters

Protein name	Human gene name	*S. saccharomyces* gene name	Main cytosolic substrate	Main matrix substrate	Reference
MCs transporting (deoxy)nucleotides					
AAC1	SLC25A4	ymr056c	ADP	ATP	Klingenberg (2008), Dolce, Scarcia, Iacopetta, and Palmieri (2005)
AAC2	SLC25A5	ybl030c			
AAC3	SLC25A6	ybr085w			
AAC4	SLC25A31				
APC1	SLC25A24	ynl083w	ATP-Mg^{2+}	P_i	Fiermonte et al. (2004)
APC2	SLC25A23				
RIM2	SLC25A33	ybr192w	Py(d)NTP	Py(d)NMP	Floyd et al. (2007), Marobbio, Di Noia, and Palmieri (2006)
PNC1					
GGC1		ydl198c	GTP	GDP	Vozza, Blanco, Palmieri, and Palmieri (2004)
NDT1		yil006w	NAD^+	(d)AMP/(d)GMP	Palmieri et al. (2009), Todisco, Agrimi, Castegna, and Palmieri (2006)
NDT2		yel006w			
CoA carrier	SLC25A42		CoA	(d)NTP, adenosine 3′,5′-diphosphate	Fiermonte, Paradies, Todisco, Marobbio, and Palmieri (2009)
MCs transporting carboxylic acid metabolites					
CIC	SLC25A1	ybr291c	Citrate	Malate	Kaplan, Mayor, Johnston, and Oliveira (1990)

Continued

Table 8.1 MCF members that are typical antiporters—cont'd

Protein name	Human gene name	*S. saccharomyces* gene name	Main cytosolic substrate	Main matrix substrate	Reference
YHM2		ymr241w	α-Ketoglutarate	Citrate	Castegna et al. (2010)
OGC	SLC25A11		Malate (α-ketoglutarate)	α-Ketoglutarate (malate)	Fiermonte, Walker, and Palmieri (1993)
ODC1	SLC25A21	ypl134c	Oxoadipate	α-Ketoglutarate	Fiermonte et al. (2001), Palmieri, Agrimi et al. (2001)
ODC2		yor222w			
DIC	SLC25A10	ylr348c	Malate, P_i	Malate, P_i	Fiermonte, Palmieri et al. (1998), Palmieri et al. (1996, 2008)
SFC1		ygr095w	Succinate	Fumarate	Palmieri, Lasorsa et al. (1997)
OAC1		ykl120w	Oxaloacetate	Sulfate/α-isopropyl-malate	Marobbio, Giannuzzi, Paradies, Pierri, and Palmieri (2008), Palmieri, Vozza, Agrimi et al. (1999)
DTC (in plant)			Oxaloacetate	Citrate, oxaloacetate, α-ketoglutarate	Picault, Palmieri, Pisano, Hodges, and Palmieri (2002)
MCs transporting amino acids or their derivatives					
AGC1	SLC25A12	ypr021c	Glutamate	Aspartate	Cavero et al. (2003), Palmieri, Pardo et al. (2001)
AGC2	SLC25A13				
ORC1	SLC25A15	yor103c	Ornithine	Citrulline	Fiermonte et al. (2003), Hoyos et al. (2003), Palmieri, De Marco et al. (1997)
ORC2	SLC25A2		H^+ (*S. cerevisiae*)	Ornithine (*S. cerevisiae*)	
SAMC	SLC25A26	ynl003c	*S*-Adenosyl-methionine	*S*-Adenosyl-homocysteine	Agrimi et al. (2004), Bouvier et al. (2006), Marobbio, Agrimi, Lasorsa, and Palmieri (2003), Palmieri, Arrigoni, et al. (2006)

characteristics, the transport driving forces *in vivo* are more complex because other factors come into play, such as the proton motive force in different respiratory states and the steady-state concentrations of all substrates, inhibitors, and/or regulators in the matrix and intermembrane space. Another factor that makes the *in vitro* and *in vivo* transport conditions different is that the MCs are in general oriented randomly in proteoliposomes, whereas they are all oriented with the N and C termini in the intermembrane space in mitochondria.

Some MCs are not obligatory antiporters. There is a group of MCs that principally catalyzes uniport transport, such as uncoupling protein 1 (Klingenberg & Winkler, 1985) and the yeast thiamine pyrophosphate carrier (Marobbio et al., 2002), or can catalyze uniport, such as the carnitine/acylcarnitine carrier (CAC) (Iacobazzi, Naglieri, Stanley, Wanders, & Palmieri, 1998; Indiveri et al., 2011; Indiveri, Tonazzi, & Palmieri, 1990, 1991; Indiveri, Tonazzi, Prezioso, & Palmieri, 1991; Palmieri, Lasorsa et al., 1999). Moreover, other MCs are symporters, including the PiC (Dolce, Iacobazzi, Palmieri, & Walker, 1994; Fiermonte, Dolce, & Palmieri, 1998; Kadenbach, Mende, Kolbe, Stipani, & Palmieri, 1982; Runswick, Powell, Nyren, & Walker, 1987) and the glutamate carriers GC1 and GC2 (Fiermonte et al., 2002). In this review, we focus on the MCs that function as antiporters as defined by *in vitro* experiments, and discuss their mode of transport and mechanism.

2. TRANSPORT MODE OF THE MCF ANTIPORTERS

In this section, some MCF members that most likely function as antiporters *in vivo* are described. They exemplify four different transport modes with respect to their dependency on the proton motive force. Transport activities across the mitochondrial inner membrane may be electroneutral or electrophoretic (electrogenic) (i.e., independent or dependent on $\Delta\psi$, respectively), as well as dependent or independent on ΔpH.

2.1. The ADP/ATP carrier

The mitochondrial matrix is the site of oxidative phosphorylation, where ATP is produced from ADP and P_i by ATP synthase in a mechanism involving the respiratory chain complexes and the proton motive force. The matrix-produced ATP is exchanged for cytosolic ADP by the AACs (also called adenine nucleotide translocators) at a ratio of 1:1 (Klingenberg,

2008; Pfaff & Klingenberg, 1968; Pfaff, Klingenberg, & Heldt, 1965). Cytosolic ATP is consumed by a large number of processes and the products ADP and P_i are recycled to the matrix by the AACs and the PiC, respectively. The transport step of the latter carrier costs one proton pumped by the respiratory chain complexes per ATP synthesized (Watt, Montgomery, Runswick, Leslie, & Walker, 2010). The AACs have a narrow substrate specificity because they transport only ADP and ATP. These transporters are inhibited by the specific inhibitors atractyloside and bongkrekic acid (Klingenberg & Buchholz, 1973). The four human AACs have different tissue distributions: AAC1 is found in heart and skeletal muscle, AAC2 is found in proliferating cells, AAC3 is ubiquitous, and AAC4 is found in lung and testis (Dolce et al., 2005; Stepien, Torroni, Chung, Hodge, & Wallace, 1992). They have a K_m for ADP in the micromolar range (De Marcos Lousa, Trézéguet, Dianoux, Brandolin, & Lauquin, 2002), except for AAC4 that has a K_m approximately 10-fold higher.

The $\Delta\psi$ and ΔpH dependencies of the ADP/ATP exchange catalyzed by the mitochondrial AACs have been investigated by using two different ionophores—valinomycin and nigericin (LaNoue, Mizani, & Klingenberg, 1978; Villiers, Michejda, Block, Lauquin, & Vignais, 1979). An artificial $\Delta\psi$ can be created across the membrane of isolated mitochondria or proteoliposomes by adding KCl externally and the K^+-specific ionophore valinomycin (Fig. 8.2A). Valinomycin allows K^+ to pass across the membrane in the direction of the concentration gradient (i.e., inward), thereby creating a charge separation because the Cl^- counter ions remain on the outside. Under these conditions, if transport is dependent on $\Delta\psi$, then the exchange rate of an MC carrying unevenly charged substrates from opposite sides of the membrane should be affected. If a net positive charge is transported inward (or a net negative charge outward) by the exchange, then the transport rate is decreased by the applied $\Delta\psi$. If, on the other hand, a net positive charge is transported outward (or a net negative charge inward), then the transport rate is increased.

An artificial ΔpH gradient can be created across isolated mitochondria or proteoliposomes by the addition of KCl and nigericin on the outside (Fig. 8.2B). Nigericin allows both K^+ and H^+ to pass across the membrane by an exchange mechanism. Because $[K^+]$ is high on the outside and there is a concentration gradient across the membrane, nigericin will in fact exchange external K^+ for internal H^+. In this way, no $\Delta\psi$ is created; rather, a ΔpH with higher $[H^+]$ on the outside is created by the transfer of protons outward.

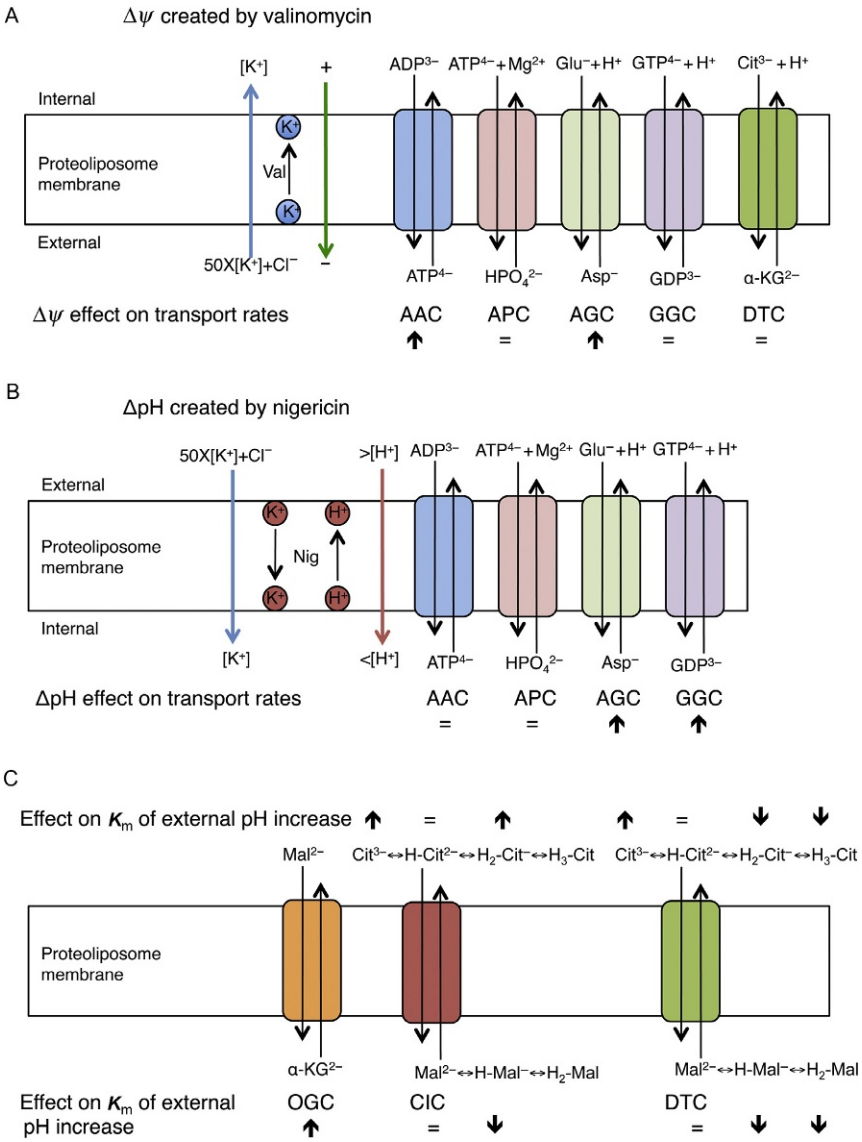

Figure 8.2 Use of valinomycin and nigericin to generate $\Delta\psi$ and ΔpH in proteoliposome transport assays. (A) External addition of KCl and valinomycin (Val, blue filled circles) allows K^+ to pass across the proteoliposome membrane inwardly (blue arrow) while Cl^- remains outside. In this way, the charges are separated across the membrane resulting in a positive net charge internally and a net negative charge externally (green arrow). The effects of $\Delta\psi$ on the transport rates of some antiporters are indicated by equal signs (unaffected) and arrows pointing up (increase). (B) A pH gradient across the proteoliposome membrane can be created by transforming the K^+

(Continued)

Under these experimental conditions, the ADP/ATP exchange catalyzed by the AACs was shown to be dependent on the $\Delta\psi$, but not on the ΔpH *in vitro* (Dolce et al., 2005; LaNoue et al., 1978; Villiers et al., 1979). Therefore, the transport mechanism is electrophoretic, that is, the net negative charge that is transferred from the matrix to the cytosol in the ADP^{3-}/ATP^{4-} exchange moves down the transmembrane charge gradient (Fig. 8.2). These conclusions from the ionophore experiments were confirmed by measuring electrical currents that arise upon ADP/ATP exchange by AAC in reconstituted vesicles (Brustovetsky, Becker, Klingenberg, & Bamberg, 1996; Gropp et al., 1999). These findings suggest that a major driving force for the exchange of cytosolic ADP^{3-} for matrix ATP^{4-} *in vivo* comes from the $\Delta\psi$, which is high in energized animal cells. However, the transport rates *in vivo* also depend on the cytosolic and matrix [ATP] and [ADP]. Active cells are thought to have ATP and ADP concentrations of about 5 and 1 mM, respectively, and the [ATP]/[ADP] ratio fluctuates only slightly despite extreme changes in the energy demand. The AACs work constantly toward equilibrating steady-state concentrations of ADP and ATP between the matrix and the cytosol. The effective ATP concentration could be influenced by Mg^{2+} because of its ability to complex ATP, and the Mg^{2+} concentration might be different inside the matrix with respect to the cytosol.

2.2. The ATP-Mg^{2+}/P_i carrier

The matrix concentration of adenine nucleotides can change upon increased demand for ATP-consuming processes, such as gluconeogenesis from lactate and mitochondrial transcription. Under these conditions, mitochondria require net transport of adenine nucleotides across the inner membrane besides the exchange of mitochondrially produced ATP for cytosolic ADP catalyzed by the AACs. This net transport is catalyzed by the

Figure 8.2—Cont'd gradient (blue arrow) into a H^+ gradient (red arrow) by adding nigericin (Nig, red filled circle). K^+ is preferred by the ionophore on the outside because the relative $[K^+]/[H^+]$ is high, while more H^+ is preferred on the inside because the relative $[K^+]/[H^+]$ is lower. The effects of ΔpH on the transport rates of some antiporters are indicated by equal signs (unaffected) and arrows pointing up (increase). Note that the orientation of the proteoliposome membrane is switched compared to that in panel (A). (C) The effects of changing the external pH on the apparent K_m values of the OGC, CIC, and DTC are indicated by equal signs (unaffected), or arrows pointing up (increase) or down (decrease).

ATP-Mg^{2+}/P_i carrier (APC), which exchanges ATP-Mg^{2+} for P_i (Aprille, 1993; Fiermonte et al., 2004), and by the PiC, which recycles back the P_i counter-exchanged with the adenine nucleotides. In man there are four APC isoforms: APC1 (SLC25A24), which is exclusively expressed in testis; APC2 (SLC25A23) and APC3 (SLC25A25), which are almost ubiquitously expressed, although the former is preferentially expressed in kidney, liver, skeletal muscle, and heart, and the latter in lung and brain (Fiermonte et al., 2004); APC4 (SLC25A41), which is expressed in brain, testis, and liver (Traba, Satrústegui, & del Arco, 2009). All human APCs have been localized to mitochondria. APC1-3 have soluble N-terminal EF-hand Ca^{2+}-binding domains, which are found in this subfamily of the MCF and in AGC1 and AGC2; they are thought to regulate the transport activity of the C-terminal domain depending on the cytosolic Ca^{2+} concentration. Therefore, these carriers are also called Ca^{2+}-dependent MCs.

The substrates transported by human APC1 and APC2 were identified by expressing the proteins in *Escherichia coli* and reconstituting them from inclusion bodies into liposomes that were used in transport experiments with radioactive substrates (Fiermonte et al., 2004). APC1 and APC2 are strict antiporters that can exchange the substrates ATP, ADP, AMP, and P_i, and to a much lesser extent dATP, dADP, dAMP, 3′-AMP, 3′,5′-ADP, and pyrophosphate. The transport of ATP is enhanced in the presence of Mg^{2+}. The K_m values of APC1 and APC2 are very similar for the substrates ATP, ADP, AMP, P_i, and ATP-Mg^{2+} (0.3, 0.4, 1, 1.5, and 0.2 mM, respectively). The values indicate that APC1 and APC2 have the highest affinity for ATP-Mg^{2+}.

The transport mode of APC1 and APC2 was demonstrated to be electroneutral for the ATP-Mg^{2+}/P_i exchange (Fiermonte et al., 2004). The initial transport rates of ATP-Mg^{2+} in exchange for ATP-Mg^{2+}, ATP, and P_i in APC-reconstituted liposomes were unaffected when a KCl/valinomycin-generated K^+ gradient was applied across the membrane, indicating that the substrate exchange is not electrophoretic and therefore independent of the $\Delta\psi$. With the pH gradient across the APC proteoliposome membranes generated by KCl and nigericin, the transport rates for the $ATP-Mg^{2+}{}_{ex}/ATP_{in}$ exchange decreased and for the $ATP_{ex}/ATP-Mg^{2+}{}_{in}$ and ATP_{ex}/P_{in} exchanges increased; substrate homoexchanges and the $ATP-Mg^{2+}{}_{ex}/P_{in}$ rates did not change. These results suggest that the inward-directed H^+ gradient, which exists in respiratory active mitochondria, facilitates the electrically unbalanced heteroexchanges

of ATP_{ex}/P_{in} and $ATP_{ex}/ATP-Mg^{2+}{}_{in}$ by protons compensating and neutralizing the charge difference. The findings also suggest that the exchange of the major substrates, $ATP^{4-}+Mg^{2+}$ and $HPO_4{}^{2-}$, transported by the APCs (in the direction dependent on the increased or decreased mitochondrial requirement for ATP) is electroneutral (Fig. 8.2), and therefore independent not only of the $\Delta\psi$ but also of the ΔpH.

2.3. The aspartate/glutamate carrier

In glycolysis and other cytoplasmic catabolic pathways, NAD^+ is used as an electron acceptor forming NADH that needs to be reoxidized and recycled as a reductant by complex I in the mitochondrial matrix or by the glycerol-3-phosphate shuttle in the cytosol and intermembrane space in specific tissues. Because there is no MC transporting NADH directly to provide complex I with its substrate, the reducing equivalent of NADH is transported through the malate/aspartate shuttle that involves aspartate/glutamate exchange catalyzed by AGC and α-ketoglutarate/malate exchange catalyzed by OGC. The malate/aspartate shuttle has been reconstituted with purified carriers (Indiveri, Krämer, & Palmieri, 1987). The two human carriers AGC1 (also called aralar1) and AGC2 (also called citrin) have four EF-hand Ca^{2+}-binding motifs in their soluble N-terminal domains (Palmieri, Pardo, et al., 2001). AGC1 is expressed in heart, skeletal muscle, and brain, while AGC2 is expressed in many tissues, particularly the liver (Iijima et al., 2001).

Human AGC1 and AGC2 have been overexpressed in *E. coli*, purified from inclusion bodies and reconstituted into liposomes for functional characterization (Palmieri, Pardo, et al., 2001). Both carriers specifically transport aspartate, glutamate, and cysteinesulfinate by an exchange mechanism, and are regulated by Ca^{2+} binding to the intermembrane space EF-hand domains that activate transport. The K_m values of both AGC1 and AGC2 are about 0.05 and 0.2 mM for aspartate and glutamate uptake, respectively, while the V_{max} is about fourfold higher for AGC2 (about 200 μmol/min/g protein) than AGC1.

The transport mode of AGC was demonstrated to be electrogenic (Palmieri, Pardo, et al., 2001). The transport rates of homoexchanges of glutamate and aspartate in AGC1- and AGC2-reconstituted liposomes with an inward-directed K^+ gradient in the presence of valinomycin were unaffected, while the transport rate of $glutamate_{ex}/aspartate_{in}$ exchange was decreased and that of $aspartate_{ex}/glutamate_{in}$ was increased. These results

indicate that the heteroexchange is electrophoretic and dependent on the $\Delta\psi$. Therefore, *in vivo* AGC1 and AGC2 are likely to catalyze the exchange of cytosolic glutamate $+ H^+$ for matrix aspartate (Fig. 8.2).

2.4. The oxoglutarate carrier

The other membrane component of the malate/aspartate shuttle, apart from AGC, is OGC, which exchanges α-ketoglutarate for malate (Bisaccia, Indiveri, & Palmieri, 1985; Iacobazzi, Palmieri, Runswick, & Walker, 1992; Monné, Miniero, Iacobazzi, Bisaccia, & Fiermonte, 2013; Runswick, Walker, Bisaccia, Iacobazzi, & Palmieri, 1990). The substrate specificity of OGC was determined upon its overexpression in *E. coli* and reconstitution into liposomes followed by transport experiments. OGC also transports malonate, maleate, succinate, oxaloacetate, and 2-oxoadipate (Fiermonte et al., 1993). It has a K_m of about 55 μM for α-ketoglutarate and about 0.2 mM for malate (Indiveri, Palmieri, Bisaccia, & Krämer, 1987; Palmieri, Quagliariello, & Klingenberger, 1972). OGC is involved in the citrate (isocitrate)/oxoglutarate shuttle, the pyruvate/citrate cycling pathway, nitrogen metabolism, and gluconeogenesis from lactate, as well as the malate/aspartate shuttle (Palmieri, 2004). Human OGC is expressed at abundant levels in heart and skeletal muscle, and at moderate levels in brain, placenta, liver, kidney, and pancreas (Huizing et al., 1998).

All substrates of OGC possess two negative charges at physiological pH, and the α-ketoglutarate/malate exchange catalyzed by OGC is electroneutral and independent of pH (Indiveri, Palmieri et al., 1987; Palmieri, Quagliariello, & Klingenberger, 1972). It was shown that OGC-catalyzed α-ketoglutarate homoexchange rates were not pH sensitive. The apparent K_m of OGC for α-ketoglutarate was increased upon raising pH while the V_{max} remained constant, indicating that the substrate binding is affected rather than the transport rate. Thus, OGC transport is independent of ΔpH and $\Delta\psi$ (Fig. 8.2C).

2.5. The citrate carrier

Fatty acid biosynthesis, gluconeogenesis, and the citrate (isocitrate)/oxoglutarate shuttle require the transport of citrate across the mitochondrial inner membrane that is catalyzed by the citrate carrier (CIC) in exchange for malate (Palmieri, Stipani, Quagliariello, & Klingenberg, 1972). Rat CIC has a K_m for malate and citrate of 0.13 and 0.76 mM, respectively (Bisaccia, De Palma, Prezioso, & Palmieri, 1990). The substrate specificity of CIC also

includes phosphoenolpyruvate, *cis*-aconitase, and isocitrate, but not *trans*-aconitate or α-ketoglutarate (Bisaccia, De Palma, & Palmieri, 1989; Kaplan, Mayor, & Gremse, 1995; Kaplan et al., 1990). Human CIC is expressed in liver, kidney, pancreas, and in other tissues to a lesser extent (Huizing et al., 1998).

The citrate transport catalyzed by CIC was suggested to be dependent on ΔpH because CIC purified from rat liver mitochondria and reconstituted in liposomes displayed an uptake rate for citrate that was considerably higher at pH 7 than at pH 8 (Bisaccia, De Palma, Dierks, Krämer, & Palmieri, 1993). This pH dependency could be explained by an effect either on the carrier or on the substrate. The K_m for citrate transport was measured at a pH range from 7.0 to 7.8, and the K_m values for the differently charged citrate species were calculated based on the pK_a values for citrate (Bisaccia et al., 1993). The analysis showed that the K_m for citrate^{2-} remained constant in the pH range unlike the other species, suggesting that the effect was linked to protonation of the substrate. The same approach was used for CIC transport of malate and the results showed a constant K_m for malate^{2-}. Based on these results, it was therefore suggested that CIC catalyzes an electroneutral exchange of $H^+ + \text{citrate}^{3-}$ for malate^{2-}, an exchange that is therefore dependent on ΔpH (Fig. 8.2C).

2.6. The plant dicarboxylate–tricarboxylate carrier

Fatty acid synthesis, nitrogen assimilation, and the shuttling of reducing equivalents in plants are processes thought to involve the dicarboxylate–tricarboxylate carrier (DTC) that shares a substantial sequence identity with OGC but displays a broader substrate specificity (Picault et al., 2002). DTC from *A. thaliana* and *Nicotiana tabacum* was expressed in *E. coli*, purified, reconstituted into liposomes, and shown to transport both dicarboxylates (such as malate, oxaloacetate, oxoglutarate, and maleate) and tricarboxylates (such as citrate, isocitrate, *cis*-aconitate, and *trans*-aconitate). The K_m of DTC for α-ketoglutarate, malate, and citrate is in the micromolar range. The expression of DTC is found in all plant tissues.

DTC transport of citrate and α-ketoglutarate is pH dependent. The K_m values for the different charged species of citrate and malate were calculated from kinetic transport experiments at different pH values, demonstrating that the K_m was constant for the species with two negative charges (Picault et al., 2002). This finding suggests that $H^+ + \text{citrate}^{3-}$ and malate^{2-} are the main substrates for DTC (Fig. 8.2C). Furthermore, the influence of

the membrane potential on DTC transport in proteoliposomes was investigated by applying a K^+ diffusion potential in the presence of valinomycin (Fig. 8.2A). The results demonstrated that the DTC-mediated citrate/oxoglutarate exchange was independent of the $\Delta\psi$. Therefore, DTC was suggested to catalyze the electroneutral, ΔpH-dependent 1:1 substrate exchange of $H^+ +$ citrate^{3-}, α-ketoglutarate^{2-}, or malate^{2-}.

2.7. The GTP/GDP carrier (Ggc1p)

Mitochondrial GTP is required for fundamental mitochondrial processes, such as protein and RNA synthesis, as well as iron homeostasis. In many organisms, succinyl-CoA synthetase of the citric acid cycle produces GTP from GDP in mitochondria. An alternative way to generate mitochondrial GTP is by nucleoside diphosphate kinase that catalyzes the transfer of the γ-phosphate of ATP to a GDP molecule. However, in *Saccharomyces cerevisiae*, succinyl-CoA synthetase uses ADP instead of GDP (Przybyla-Zawislak, Dennis, Zakharkin, & McCammon, 1998), and nucleoside diphosphate kinase is localized in the intermembrane space (Amutha & Pain, 2003). In this species, the mitochondrial GTP/GDP carrier Ggc1p (also known as Shm1p and Yhm1p, encoded by ydl198c) catalyzes the exchange of cytosolic GTP for matrix GDP (Vozza et al., 2004).

The biochemical identification and characterization of Ggc1p was performed by expressing the protein in *E. coli* and reconstituting it from inclusion bodies into liposomes that were used in transport experiments (Vozza et al., 2004). Ggc1p is a strict antiporter that exchanges the substrates GTP, GDP, dGTP, dGDP, ITP, and IDP. ITP and IDP are structurally similar to GTP and GDP, the difference being that the former two compounds lack an amino group on the guanosine base. However, according to competitive inhibition experiments, the affinity of Ggc1p for GTP and GDP is about 10-fold higher than for dGTP and dGDP, and 100-fold higher than for IDP and ITP. This finding is also reflected by the K_m values determined for GTP, GDP, and dGTP that are 1.2, 4.5, and 15.9 μM, respectively.

The transport mode of Ggc1p was demonstrated to be electroneutral (Vozza et al., 2004). The transport rates of homo- and heteroexchanges of GDP and GTP in Ggc1p-reconstituted liposomes were unaffected by valinomycin and an inward-directed K^+ gradient, indicating that the GDP/GTP heteroexchange is not electrophoretic and therefore independent of the $\Delta\psi$. When a pH gradient was generated across Ggc1p proteoliposome membranes by adding KCl and nigericin, the transport rate

for the GDP_{ex}/GTP_{in} exchange decreased, and that for the GTP_{ex}/GDP_{in} exchange increased; the homoexchange rates were unchanged. These results suggest that the inward-directed H^+ gradient, created by the respiratory chain complexes in mitochondria, facilitates the import of $GTP^{4-} + H^+$ in electroneutral exchange with GDP^{3-}, and the Ggc1p-mediated transport is dependent on ΔpH (Fig. 8.2).

Further support for the conclusion that GTP and GDP are the main substrates for Ggc1p *in vivo* stems from studies of *GGC1Δ* yeast cells where mitochondria have increased levels of GDP and decreased levels of GTP (Vozza et al., 2004). It has been demonstrated that the lack of Ggc1p interferes with cellular iron homeostasis (Lesuisse, Lyver, Knight, & Dancis, 2004)—a defect that can be complemented by the expression of the human mitochondrial nucleoside diphosphate kinase in mitochondria of *GGC1Δ* yeast cells (Gordon, Lyver, Lesuisse, Dancis, & Pain, 2006). That Ggc1p also transports dGDP and dGTP *in vivo* is supported by the fact that it acts as a suppressor of a mutation leading to loss of mitochondrial DNA, and thereby plays a role in maintaining the mitochondrial genome (Kao, Megraw, & Chae, 1996).

3. TRANSPORT MECHANISM OF MCF ANTIPORTERS

3.1. Structure of MCs

For a long time, MCs were thought to be homodimeric. This hypothesis was based on quantifications of how many atractyloside molecules bind to each AAC protein, their apparent size when migrating on gel filtration columns or in blue native gels, and kinetic experiments (Aquila, Eiermann, Babel, & Klingenberg, 1978; Bisaccia et al., 1996; Block & Vignais, 1984; Block, Zaccaï, Lauquin, & Vignais, 1982; Brandolin et al., 1980; Hackenberg & Klingenberg, 1980; Lin, Hackenberg, & Klingenberg, 1980; Palmieri, Indiveri, Bisaccia, & Krämer, 1993; Palmieri, Vozza, Hönlinger et al., 1999; Palmisano et al., 1998; Riccio, Aquila, & Klingenberg, 1975; Schroers, Burkovski, Wohlrab, & Krämer, 1998). However, these results have been re-interpreted, and a large body of evidence is now pointing toward MCs being monomers (Crichton, Harding, Ruprecht, Lee, & Kunji, 2013; Kunji & Crichton, 2010). The X-ray crystallographic structure of bovine AAC1 showed that a single polypeptide chain could form a protein fold by itself, and the crystal packing did not reveal any hint of dimer formation (Pebay-Peyroula et al., 2003). Yeast AAC2 is monomeric in detergent solution and during purification (Bamber, Harding, Butler, &

Kunji, 2006; Bamber, Slotboom, & Kunji, 2007). No inhibitory effect of hypothetical dimer formation could be seen when varying concentrations of mixed active and inactive mutant forms of purified AAC2 were reconstituted into liposomes and transport assays were performed (Bamber, Harding, Monné, Slotboom, & Kunji, 2007). Furthermore, the transport activities of all the mutants used in the complete cysteine-scanning mutagenesis of OGC have revealed that no functionally crucial or conserved dimer interface is found on the bilayer-exposed surface of the OGC structural homology model (Miniero et al., 2011).

3.2. Matrix and cytoplasmic gates in MCs

The inhibitors atractyloside and bongkrekic acid trap the AACs in two different conformations known as the c- and m-state where the carrier is open to the cytoplasmic (intermembrane space) and matrix sides, respectively (Aquila et al., 1978; Brandolin, Dupont, & Vignais, 1985; Buchanan, Eiermann, Riccio, Aquila, & Klingenberg, 1976). The structure of bovine AAC1 revealed the conformation of the carrier inhibited by carboxy–atractyloside where the six-transmembrane α-helix bundle is closed toward the matrix side by a kink in H1, H3, and H5, and a salt-bridge network formed by the charged residues of the threefold repeated signature motif (Fig. 8.3A). These charged residues have been shown to be critical for transport function in AAC (Nelson, Felix, & Swanson, 1998), OGC (Cappello et al., 2007), and CAC (Giangregorio, Tonazzi, Console, Indiveri, & Palmieri, 2010). The AAC1 structure has therefore been seen as representing the carrier with a closed matrix gate as in the c-state.

On the even-numbered α-helices, charged residues have been found near the cytoplasmic side that could form a salt-bridge network closing the cytoplasmic gate (Fig. 8.3C) (Robinson, Overy, & Kunji, 2008). The charged residues of the cytoplasmic and matrix gates are generally conserved in the MCF and are symmetric, that is, amino acids with identical charge occupy the corresponding positions in all three carrier repeats. The residues of the proposed cytosolic gate in OGC have been shown to be functionally important (Miniero et al., 2011).

3.3. The substrate binding site in MCs

The bovine AAC1 structure is in an atractyloside-inhibited form. However, a substrate binding site was proposed based on its approximate location in the 3D structure, and on mutagenesis work that indicated residues crucial for

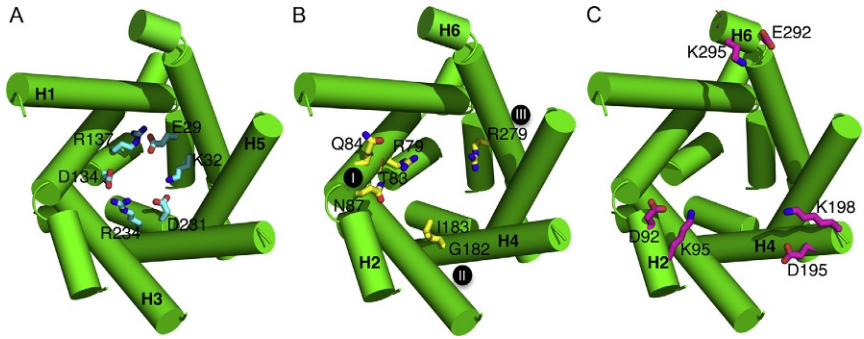

Figure 8.3 Important residues for transport in the central cavity of the bovine AAC1 structure. For simplicity, the loops and matrix α-helices have been removed. (A) The matrix gate is formed by the matrix salt-bridge network that consists of the charged residues of the first half of the signature motif on H1, H3, and H5. (B) The common substrate binding site is formed by three contact points (Roman numbered black filled circles) consisting of residues on H2, H4, and H6 above the matrix gate. (C) The hypothetical cytoplasmic gate is formed in the matrix conformation by a salt-bridge network of the charged residues on top of the three even-numbered transmembrane α-helices.

carrier function (Pebay-Peyroula et al., 2003). The definition of the substrate binding site in AAC has later been refined by using molecular dynamics simulations with ADP as a ligand in the carrier cavity (Dehez, Pebay-Peyroula, & Chipot, 2008; Falconi et al., 2006; Wang & Tajkhorshid, 2008). Despite the relatively low sequence identity among many MCs, structural homology models based on the bovine AAC1 structure can be made from reliable alignments due to the presence of the highly conserved signature motifs in all MCF members that define the beginning and the end of the even- and odd-numbered α-helices, respectively. By analyzing cavity residues that display covariation with the substrates of characterized MC subfamilies (Palmieri, Agrimi, et al., 2006 for a review), Robinson and Kunji (2006) proposed a putative common binding site (Fig. 8.3B). This binding site consists of three contact points—one on each even-numbered α-helix between the matrix and the cytoplasmic gates close to the middle of the membrane bilayer. Based on the identity of the residues in contact point II on H4, the following three major classes of substrates can be distinguished: nucleotides, carboxylates, and amino acids. The common binding site corresponds to asymmetric residues, that is, different kinds of amino acids occupy the same corresponding positions within the three tandem repeats (Robinson et al., 2008). The complete cys-scanning

mutagenesis studies of OGC, as well as the site-directed mutagenesis data of other carriers, support the crucial role of the contact point residues for function (Briggs, Mincone, & Wohlrab, 1999; Cappello et al., 2006; De Lucas et al., 2008; Giangregorio et al., 2010; Heidkämper, Müller, Nelson, & Klingenberg, 1996; Ma et al., 2007; Stipani et al., 2001; Tonazzi, Console, Giangregorio, Indiveri, & Palmieri, 2012; Wohlrab, Annese, & Haefele, 2002). Experimental evidence that residues in the common binding site directly interact with the substrate has been found in yeast AAC2 and the human ornithine carrier. The mutation R294A of contact point III in AAC2 diminishes the transport rate of ATP without affecting the transport of ADP (Heidkämper et al., 1996), and the substrate specificities of the two human ornithine carriers could be swapped by exchanging the arginine and the glutamine at contact point II of the two isoforms (Monné et al., 2012). Although the common substrate binding site may be mainly responsible for substrate binding, it does not fully explain how different substrates are selected by carriers with identical contact point residues. Other asymmetric residues that are specific for certain MC subfamilies and protrude into the internal carrier cavity in proximity to the three contact points most likely play a role in fine-tuning the substrate specificity of MCs (Palmieri & Pierri, 2010b; Palmieri et al., 2011; Pierri, Palmieri, & De Grassi, 2013). Consequently, potential substrates for an MC with unknown function can be predicted by identifying the small set of residues in its sequence that defines the binding site (Palmieri et al., 2011).

3.4. General transport mechanism for MCs

By combining the data concerning the monomeric state, the evidence for a matrix and cytoplasmic gate, and the location of the substrate binding site, it is possible to suggest a simple model for the antiport transport mechanism of MCs (Palmieri & Pierri, 2010a, 2010b; Palmieri et al., 2011; Robinson et al., 2008) within the framework of the structure of bovine AAC1. This model basically represents the "single binding center-gating pore mechanism" as proposed earlier by Klingenberg (Klingenberg, 1976, 1979) in which the substrate binding site is alternatively accessible from the cytoplasmic or the matrix side. For example, the exchange of cytosolic ADP for matrix ATP catalyzed by AACs includes the following steps (i–vi):

(i) ADP binds to the substrate binding site exposed to the intermembrane space, that is, in the c-state (Fig. 8.3B).

(ii) The ADP–carrier interactions induce conformational changes that involve opening of the matrix gate and closing of the cytoplasmic gate,

and result with the substrate bound in the m-state. The conformational change is driven by the binding energy of the substrate to the substrate binding site residues (Fig. 8.3B), and might involve the substrate interfering with the matrix salt-bridge network (Fig. 8.3A) and/or assisting the formation of the cytoplasmic salt-bridge network (Fig. 8.3C). The substrate–carrier interactions could therefore involve both the even-numbered α-helices (binding site and cytoplasmic salt-bridge network) and the odd-numbered α-helices (binding site and matrix salt-bridge network). Moreover, the opening of the matrix gate is likely to involve a switch of the interactions between the relayed charged residues. There may be an occluded state where the substrate is bound and both gates are partially closed, but exactly what the transition- and m-states look like is not known. However, the m-state is probably also pseudo-symmetric because the matrix and cytosolic gate residues are symmetric and, as suggested by the mechanism described here, all three contact points are involved in substrate translocation.

(iii) ADP leaves the substrate binding site of the carrier that is open toward the matrix side. The carrier cannot switch back to the c-state conformation without another bound substrate because it is a strict antiporter.

(iv) ATP binds to the matrix substrate binding site that most likely involves the same residues of the cytoplasmic substrate binding site, but which are positioned in a different conformation.

(v) The ATP-carrier interactions induce the reverse conformational changes leading to closure of the matrix gate and opening of the cytoplasmic gate.

(vi) ATP is released into the intermembrane space and leaves the carrier in the c-state ready for a new cycle of transport.

Mechanistically, MC uniporters and antiporters could work the same except for the requirement in the 1:1 exchange of a counter substrate to induce the retrograde conformational change (step iv). In the case of the uniporters, probably one of the gates (e.g., the cytoplasmic one) is destabilized and only forms transiently during substrate translocation; then it is reopened and the carrier switches directly back to the outside open state without the bound substrate (steps iv–vi). MC uniporters can also perform antiport with increased velocity *in vitro*, because in proteoliposomes, the orientation of the carriers is random and the high counter-substrate concentration drives the opening of the carriers toward the outside.

Thus, the mechanism described here suggests a single substrate binding site in MCs that switches between two or three major conformations, that is,

with the binding site open toward the cytosol or the matrix, or occluded from both sides in the transition state. Mutagenesis data from many MCs may be interpreted within the context of the substrate binding site and the transport mechanism as described above (Monné, Palmieri, & Kunji, 2013). Moreover, most of the disease-causing single point mutations are found along the carrier substrate translocation path (Palmieri, 2008; Poduri et al., 2013; Wibom et al., 2009). In the structural homology models of MCs, there are two levels in the carrier cavity where glycines and prolines are symmetric and conserved in transmembrane α-helices (Palmieri & Pierri, 2010b). These residues have been suggested to work either as sites for close helix–helix interactions (Robinson et al., 2008) or as hinges required for opening and closing the cytoplasmic and matrix gates (Palmieri & Pierri, 2010b).

It should be noted that because the transport mechanism described above suggests a single substrate binding site that can bind the substrate in two different conformations, the affinities (K_m) for the same substrate on the cytosolic and matrix sides might be different, as experimentally observed with some carriers in reconstituted liposomes (Palmieri, Indiveri, Bisaccia, & Krämer, 1993). These different substrate affinities will help to maintain different steady-state concentrations of the substrate in the cytosol and in the matrix. In the case where a difference in substrate concentration is not required by the cell, the cytosolic and matrix K_m is probably close to equal.

In the case of carriers that are dependent on ΔpH, it is not completely clear if the proton binds to a residue in the binding site or protonates the substrate. The protonation is probably allowed by the pH gradient and the environment within the substrate binding site causing alteration of local pK_a values. Residues involved in proton coupling have been hypothesized (Kunji & Robinson, 2010), but remain to be established experimentally.

4. KINETIC MECHANISM OF MCs

Several MC antiporters that represent a two-substrate reaction (one substrate binding to the carrier on the outside and one on the inside of the membrane) have been studied kinetically. Most MCs seem to operate according to a sequential (simultaneous) mechanism (Bisaccia et al., 1993; Dierks, Riemer, & Krämer, 1988; Indiveri, Dierks, Krämer, & Palmieri, 1991; Indiveri, Prezioso, Dierks, Krämer, & Palmieri, 1993; Indiveri, Tonazzi, De Palma, & Palmieri, 2001; Sluse, Duyckaerts, Liebecq, & Sluse-Goffart, 1979; Sluse et al., 1991; Stappen & Krämer, 1994), suggesting that both the cytoplasmic and matrix substrates first bind to the carrier (i.e.,

form a ternary complex) before they are translocated. These results are consistent with the idea that MCs are homodimers with one protomer open toward the cytosol and the other toward the matrix. Upon the binding of the substrate to each protomer (on opposite sides), cooperative conformational changes are induced leading to the exchange of the substrates between the two compartments (Palmieri, Indiveri et al., 1993).

However, the formation of a ternary complex is incompatible with the general transport mechanism of monomeric MCs as described above that corresponds kinetically to a ping-pong mechanism. In this mechanism, one substrate binds to the open carrier and is then translocated across the membrane leaving the carrier open on the opposite side ready for the counter substrate to bind and be translocated. In other words, the substrates are dependent on each other for the conformational change but do not form a ternary complex with the carrier. The only MC found to follow a ping-pong mechanism is CAC (Indiveri, Tonazzi, & Palmieri, 1994)—a finding that might reflect the fact that CAC is able to catalyze uniport transport as well as exchange of substrates. Because of the apparent contradiction between the proposed transport mechanism and the kinetic data, it is possible that either the model for the transport mechanism or the interpretation for the sequential mechanism of MCs is incorrect.

5. SYNOPSIS

Many MCs have been known to be antiporters for a long time, although how they perform this function at the molecular level was unclear. The current model for the transport mechanism of MCs is based on the wealth of information now available about the MCF, including biochemical characterization of a large number of carriers, site-directed mutagenesis studies, identification of disease-causing mutants, genomic sequences, sequence analysis, and structural and functional studies. The picture of the transport mechanism that has emerged applies in part to all MCs and in part to each MC subfamily. A single similarly located binding site and a matrix and cytoplasmic gate are involved in substrate translocation of all MCs by an alternating access mechanism. Specific residues of the binding site, specific substrate–carrier interactions, regulation by N-terminal Ca^{2+}-binding domains, dependency on $\Delta\psi$ and ΔpH, as well as anti-, uni-, and symport modes of transport vary among MC subfamilies. In other words, the driving forces and energies vary, but the structural scaffold and probably also the substrate translocating conformational changes are fundamentally the same for all MCs.

Future investigations are warranted to provide further evidence for the current transport mechanism of MCs and to refine it. The determination of MC structures in the m-state and intermediate conformations within the transport cycle is imperative to assess the dynamics involved in substrate translocation. Many MCF members have not been characterized yet, and several substrates known to be transported across the mitochondrial inner membrane have not been assigned to a transporter. In this respect, the recent finding that uncoupling protein 2 is a transporter for four-carbon metabolites (Vozza et al., 2014) strongly suggests that the function of the previously-named uncoupling proteins 3-5 (encoded by SLC25A9, SLC25A14 and SLC25A27) can also be to transport solutes across the mitochondrial membrane. All the above-mentioned knowledge could contribute to the understanding of the physiological roles of MCs and the molecular basis for the deleterious effects of the constantly increasing number of mutations in MCs involved in human disease.

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