Acta Biochimica Polonica

Vol. 43 No. 2/1996

349–360

QUARTERLY

Lecture delivered at the International Conference on "New Frontiers in Cell and Molecular Biology" held in Warsaw (Poland) on October 2–7, 1995

Mitochondrial metabolite carrier family, topology, structure and functional properties: an overview

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Key words: mitochondrial metabolite; carrier family

A set of metabolite carriers operates the traffic of numerous molecules consumed or produced in mitochondrial matrix and/or cytosolic compartments. As their existence had been predicted by the chemiosmotic theory, the first challenge, in the late sixties, was to prove their presence in the inner mitochondrial membrane and to describe the various transports carried out. The second challenge was to understand their mechanisms by the kinetic approach in intact mitochondria (seventies). The third challenge (late seventies–eighties) was to isolate and to reconstitute the carriers in liposomes in order to characterize the proteins and to establish the concept of a structural and a functional family as well as some structure-function relationship with the help of primary sequences. Genetics, molecular biology and genomic sequencing bring the fourth challenge (nineties): a raising number of putative carriers becomes known only by their primary sequences but their functions have to be discovered. The actual challenge of the future is the elucidation of the ternary structure of carrier proteins that together with site-directed mutagenesis and kinetic mechanism will permit to advance in the understanding of molecular mechanisms of transport processes.

The genius of chemiosmotic concept of energy conservation proposed by Peter Mitchell in 1961 [1] implied first, the presence of an isolating membrane allowing to build, by the proton pumps of the respiratory chain, a proton electrochemical potential across this membrane and, second, the occurence of a proton circuit coupling the redox reactions of the respiratory chain to the synthesis of ATP by ATP synthase. The acceptance of the chemiosmotic theory obviously implied that the transport of metabolites and more generally of ions through the inner mitochondrial membrane requires a battery of specific carriers.

The first challenge, in the middle sixties, was to prove the presence of these carriers in the inner mitochondrial membrane and to describe the various transports carried out. The pioneering studies of transport were performed by B. Chappell [2] and E. Pfaff [3, 4]. Nowadays, there are thirteen well characterized metabolite carriers in mitochondria (for a recent review see [5]). These carriers transport anions such as ADP/ATP, inorganic phosphate, pyruvate, glutamate, aspartate, oxoglutarate, dicarboxylate, citrate, ATP·Mg/P_i, but also cations or zwitterions such as ornithine, carnitine, proton and glutamine. Beside these well defined "major" carriers, numerous others must exist for the import of various compounds which are not synthesized in the mitochondrial matrix such as arginine, proline, thiamine-PP, gluta-

Abbreviations: MCF, mitochondrial carrier family; see also Table 1.

thione, choline, various nucleotides, biotin, CoA etc. However, these "minor" carriers have not been well characterized yet. Moreover, genetic studies and genomic sequencing have allowed to identify genes coding for proteins that are putative mitochondrial carriers with unknown function. All these carriers, major, minor and putative, constitute a protein family called MCF (for Mitochondrial Carrier Family) [6, 7].

It was quite easy to reach the first level of understanding of the transport processes (Table 1) that deals with the features of the process as uniport, symport and antiport (or exchange), with their stoichiometries and their driving force characteristics as electrophoretic, electroneutral H^+-compensated, electroneutral or neutral (for a review and references see [8]). However, for the next level of understanding, dealing with mechanistic and molecular properties, the progress was very slow. Nevertheless, significant advancements have been made thanks to the hard work of a few laboratories [9] that have succeeded in bringing this field to the molecular level by elucidating their kinetic mechanisms, purifying and reconstituting the major carriers, elucidating an increasing number of primary sequences, investigating the transmembrane topology, expressing some of them in yeast and *E. coli* and reaching the mutational analysis stage as well as gene-expression regulation.

The ultimate aim in carrier research is to describe the sequence of intimate interactions between the translocated molecules and the carrier protein allowing the movement of the solute across the membrane, i.e., the molecular mechanism of transport catalysis. This level of understanding lies on three types of approaches, at first sight independent, but their results have to be analyzed in a concerted way: (1) the functional studies by means of detailed kinetic studies leading to kinetic mechanisms, (2) the molecular studies including chemical modification, determination of primary sequence, prediction of secondary structures, transmembrane topology, three dimensional structure description, and (3) the genetic studies with gene identification, genomic sequencing, over-expression and site directed mutagenesis.

Table 1

Transport mode, substrates and driving force of some major carriers.

$\Delta\psi$ is the transmembrane electrical potential and ΔpH is the pH gradient across the membrane.

Carrier species (abbreviation)	Substrates	Transport mode	Driving force
Electrophoretic			
Adenine nucleotide (AAC)	ADP, ATP	antiport	$\Delta\psi$
Aspartate/Glutamate (AGC)	Aspartate, Glutamate	antiport	$\Delta\psi + \Delta$pH
Uncoupling protein (UCP)	Proton	uniport	$\Delta\psi + \Delta$pH
Electroneutral H^+ compensated			
Phosphate (PiC)	Phosphate/hydroxyl	symport/antiport	ΔpH
Pyruvate (PYC)	Pyruvate/hydroxyl	symport/antiport	ΔpH
Glutamate	Glutamate/hydroxyl	symport/antiport	ΔpH
Electroneutral			
Oxoglutarate (OGC)	Oxoglutarate, Malate, Succinate	antiport	–
Dicarboxylate (DIC)	Malate, Phosphate	antiport	–
Tricarboxylate (CIC)	Citrate, Malate	antiport	–
Ornithine (ORC)	Ornithine	antiport	–
Neutral			
Carnitine (CAC)	Carnitine/acylcarnitine	antiport/uniport	–
Glutamine	Glutamine	uniport	–

The aim of this overview is to stress the convergent informations resulting from the various approaches that have enriched the understanding of the mitochondrial carriers and to bring out the future challenges of the field.

KINETIC MECHANISMS

At the very beginning of the seventies, the first attemps to determine the kinetic mechanism of a metabolite carrier were undertaken in intact mitochondria. The oxoglutarate carrier of heart mitochondria was the easiest tool, because it is the only carrier in these mitochondria that catalyses electroneutral exchanges between oxoglutarate, malate, malonate and succinate [10]. Two-substrate initial-rate studies have led to a kinetic pattern in double-reciprocal plots ([initial rate]$^{-1}$ *versus* [concentration]$^{-1}$ of both translocated substrates) that shows convergences on abscissa axis whatever the counter substrate of the exchange is [11]. This kinetic pattern means that the oxoglutarate carrier functions according a double-binding site mechanism with independent binding of the internal and the external substrates. Then both substrates form randomly a ternary complex with the carrier (internal and external binding sites are simultaneously accessible) before the transport (rate limiting step) occurs [12].

Very extensive kinetic studies of the oxoglutarate carrier in intact mitochondria have demonstrated that the oxoglutarate carrier is an oligomeric association of functional subunits. A functional subunit (Fig. 1) is constituted of an external binding domain, a catalytic domain within a channel and an internal binding domain. Binding of external oxoglutarate induces a conformational change of the external domain whereas the binding of internal malate does not. The central catalytic part is not modified by the substrate bindings, so in any case

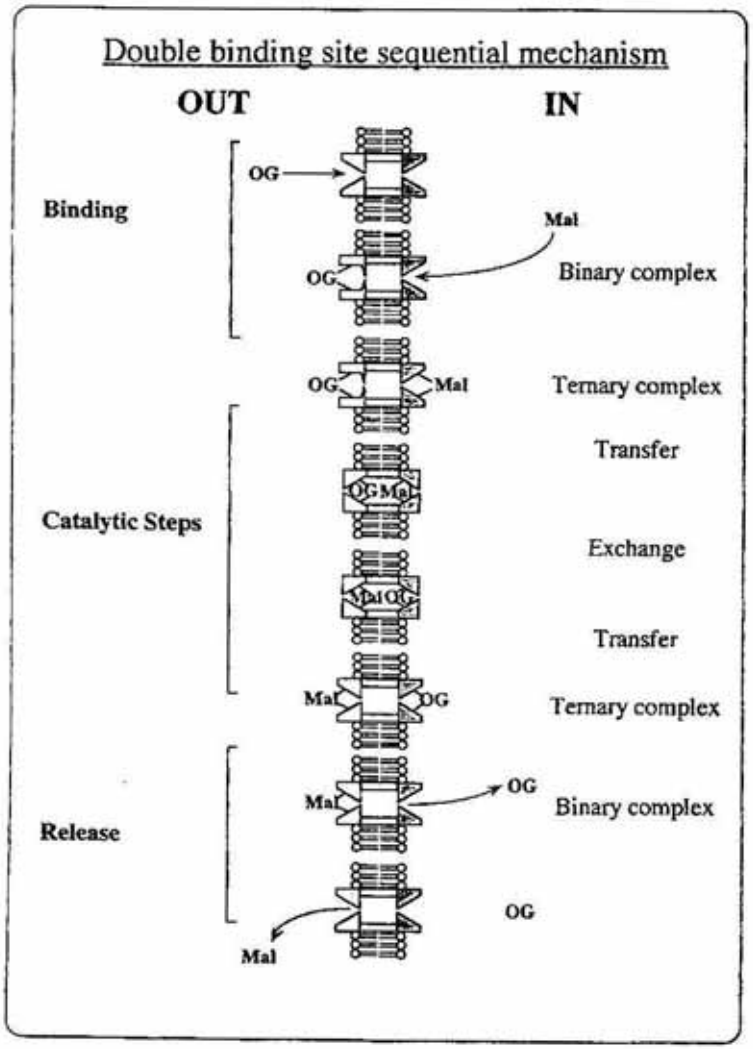

Fig. 1. Example of double-binding site mechanism: the sequential (or simultaneous) mechanism of the oxoglutarate carrier exchanging external oxoglutarate (OG) against matricial malate (Mal) is represented.
The channel is in white and the binding domains in grey. The conformational changes occuring during the catalytic cycle (exchange) are schematically represented for each step.

the two substrates bind independently. Thus there is no long-distance interaction. The catalytic step begins with a transfer of the two substrates to the sites in the channel and is accompanied by another conformational change of the two surface binding domains. The next step is the exchange of the two substrates within the channel. It is the rate limiting step. Complete reaction involves the transfer of the products to the surface parts and their release into the aqueous solutions with a return to the starting conformations of the carrier [13–16].

This mechanism is in complete opposition to the single-binding site or ping-pong mechanism proposed by Klingenberg for ADP/ATP carrier in 1973 [17–19]. This mechanism does not involve a ternary complex because the carrier has either one site accessible from the outside or one site accessible from the inside (Fig. 2). The transition from one form to the other implies the transport of one substrate, either the import of the external substrate or the export of the internal substrate. This mechanism was proposed on the basis of the competitive binding studies between ADP, ATP and two specific inhibitors, carboxyatractylate (binding on the carrier only from the outside) and bongkrekate (binding only from the inside). In fact, these binding studies have only shown that the adenylic carrier has two different conformations that are stabilized by the inhibitory ligands. No mechanistic properties can be deduced from such binding studies. Indeed, the same type of binding studies performed by the group of Vignais on isolated carrier and on carrier in native membrane have suggested that the carrier is organized as a tetramer. Each tetrameric carrier would contain two carboxyatractylate-binding sites accessible from the cytosolic face, or two bongkrekate-binding sites accessible from the matrix and mostly four nucleotide binding sites two on each face of the membrane (for a review see [20]).

Two-substrate initial rate studies carried out since 1988 with reconstituted carriers in liposomes have demonstrated the occurence of double binding site sequential mechanisms for

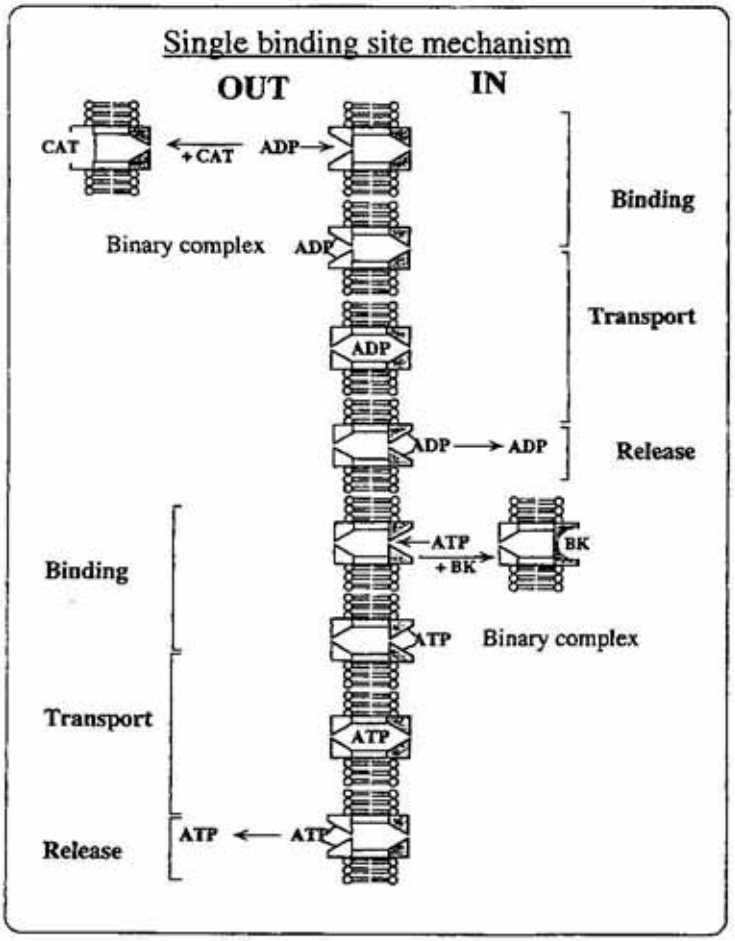

Fig. 2. Example of single-binding site mechanism: the ping-pong mechanism is illustrated by the ADP/ATP exchange.

The two conformations stabilized by the external carboxyatractylate (CAT) and by the internal bongkrekate (BK) are represented as well as the steps of the successive transport of external ADP and the internal ATP.

Table 2
Kinetic mechanisms of metabolite carriers

Carrier	Materials	Kinetic mechanism	References and year
AAC	Reconstituted Mitochondria	n.d. sequential	[21] 1982 [22] 1980, [23] 1989
AGC	Reconstituted Mitochondria	sequential sequential	[24] 1988 [25] 1991
UCP	–	–	–
PiC	Reconstituted	sequential	[26] 1990
PYC	Reconstituted Mitochondria	sequential n.d.	[27] 1994 [28] 1994
OGC	Reconstituted Mitochondria	sequential sequential	[29] 1991 [11] 1972
DIC	Reconstituted	sequential	[30] 1993
CIC	Reconstituted	sequential	[31] 1993
ORC	Reconstituted	n.d.	[32] 1994
CAC	Reconstituted (liver) Reconstituted (brain)	ping-pong sequential	[33] 1994 [34] 1995

all the studied carriers (Table 2). They irrefutably confirm the conclusions of the pioneering kinetic study of oxoglutarate carrier in intact mitochondria (for a recent review see [35]). The only exception may be the carnitine carrier for which conflicting results were obtained: ping-pong mechanism in liver mitochondria [33] and sequential mechanism in brain mitochondria [34]. This discrepancy may be due to very different experimental procedures or a tissue specificity.

The mechanism of ADP/ATP carrier is not yet determined as mentioned by Palmieri [5] in 1994 because, in contrast to other carriers, its reconstitution leads to random orientation in the liposome membrane. Two-substrate initial rate studies on intact mitochondria have been started in 1980 [22, 23] and nowadays led to curves in double-reciprocal plots indicating that the ADP/ATP carrier escapes from a simple Michaelis-Menten behavior. Moreover, the absence of proportionality between curves for the different internal-substrate concentrations means that inner and outer binding sites of the carrier are not independently loaded by the substrates, in contrast to all the other carriers. A numerical analysis made with the simplest equation (a ratio of two polynomials of the second degree for internal and external substrate concentrations, Sluse *et al.*, in preparation) has led to adjusted parameters indicating that:

- (1) the carrier works according a sequential mechanism;
- (2) the carrier is made of two subunits that bind orderly the two substrates (first external then internal) to form an active ternary complex; thus, there is a very strong positive cooperativity between external and internal sites and then a long distance interaction;
- (3) if the carrier is made of two identical subunits, there is a negative cooperativity in binding between the external sites, moreover the activity of one double loaded subunit decreases if the external site of the second subunit is loaded and increases if the internal site of the second subunit is also loaded;
- (4) these kinetic results taken together with the binding results of Vignais [20], suggest that one functional subunit is made of two 30 kDa monomers that build the hydrophilic path (Fig. 3).

What is emerging from the kinetic studies is that all carriers display binding sites simultaneously on both sides of the membrane and that a ternary complex, i.e., internal substrate-external substrate-carrier is compulsory. Therefore, it is surprising that in recent review papers [36], the single binding site mechanism is still claimed to be the basic mechanism for most biological carriers despite the obvious discrepancy with the results of 25 years of kinetic studies.

MOLECULAR APPROACH

Purification of carriers is essential for a molecular identification and structural studies. So far, at least 10 carriers have been purified to homogeneity from mammalian mitochondria (for extensive reviews see [8, 35, 37]) and a few from yeast. With very little exceptions, all mitochondrial carrier proteins have been purified using variations of a general procedure involving three steps adapted for the different carriers: (1) solubilization by non-ionic detergents, sometimes with high salt concentrations and addition of lipids, (2) chromatography on hydroxyapatite with, depending on the carrier, large variation of the protein/hydroxyapatite ratio or particular pretreatment of the hydroxyapatite or of the solubilized protein and (3) final purification using various additional chromatographic procedures. All mitochondrial metabolite carriers isolated until now fall into a narrow range of apparent molecular mass between 28 kDa and 34 kDa.

The first purpose of a carrier purification is to characterize it biochemically. The second step is a reincorporation of the carrier into liposome for functional studies. However, as a carrier-protein identification depends essentially on functional reconstitution, both purification and reconstituted-transport activity are tightly linked.

The first primary structure of a carrier protein was obtained by Klingenberg [38] in 1982 for the ADP/ATP carrier from bovine heart by amino acid analysis. The amino acid sequences of only five carriers (functionaly identified) have been described by amino-acid analysis or DNA sequencing, namely AAC, UCP, PiC, OGC and CIC (see Table 1 for abbreviations). Nowadays, numerous sequences of these carriers have been obtained from various organisms and therefore lead to a quite large collection from which information can be extracted. From an analysis of primary structures and from a comparison of the amino-acid sequences of these carriers several properties have been deduced (for extensive reviews see [6, 7]). Pairwise comparison realized by diagon plots, used to identify internal sequence identity, have shown that mitochondrial carrier proteins have a tripartite structure made up of related sequences of about 100 amino acids each. What is noticed between tandem repeats in a given carrier protein, is also observed by comparison of different carriers. Even if the amino-acid sequences of the repeats may be weakly related in the MCF, it is a reasonable assumption that each of the related sequences will be folded into the same structural element or domain [7]. This secondary structure is not known because of the absence of carrier-protein crystal, but it can be predicted from the hydropathy plots derived from sequence data. The interpretation of the hydropathy plots in

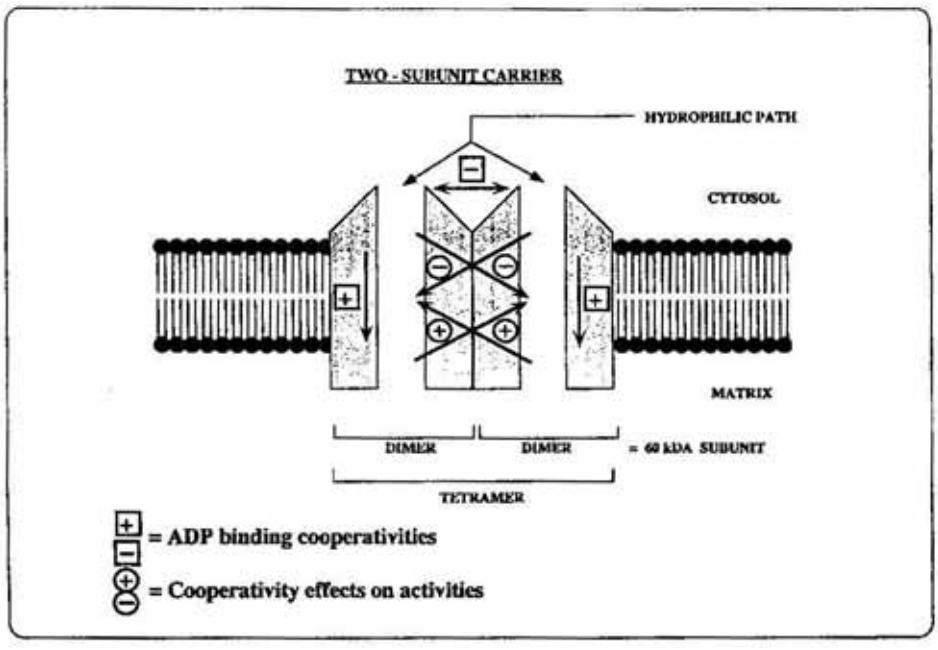

Fig. 3. Schematic representation of the ADP/ATP carrier with the kinetic and binding cooperativities: one carrier is made of two identical functional subunits (one internal and one external binding sites each) being made of two 30 kDa monomers building the hydrophilic path.
The arrows represent cooperativities, positive (+) and negative (–); the signs + or – inside a circle mean kinetic cooperativity and the signs + or – inside a square mean binding cooperativity.

spite of its uncertainty is strongly reinforced by the triplicate structure and by conserved profile in the MCF. The average hydropathy plot made by Saier [6] from 28 selected protein sequences support the view that each domain of 100 amino acids could consist of two hydrophobic transmembrane α-helices joined by hydrophilic regions even if this secondary structure is less apparent in the first repeat where the second helix is less hydrophobic. These analyses have led to a structural model that may be common for the MCF: six transmembrane α-helices cross the membrane and are connected by three long hydrophilic segments on one side of the membrane and by two short hydrophilic segments on the other side; the long loops connect the two α-helices of each repeat joined by the shorter hydrophilic loops; the N- and C-termini are situated on the same side of the membrane opposite to that of the three long hydrophilic loops (Fig. 4).

Several of these predictions are consistent with the results obtained by investigating the accessibility of polar loops of various carriers to peptide-specific antibodies, specific ligands and proteolytic enzymes that do not cross the membrane (for references see [5–7]). The N- and C-termini are exposed to the cytosolic side for PiC, UCP, CIC [39], OGC and AAC as well as loop a' for UCP and OGC. However, as the C-terminal of AAC is also accessible from the matrix side, no definite conclusion can be proposed [20]. The loops A, B and C are accessible to proteases on the matrix side for AAC and UCP, the loops A and B for OGC and the loop B for PiC.

Some photolabelling experiments have given rise to data which seem to conflict with this model. It was shown by Brandolin [20] that segments of matricial loops B and C of AAC (F153-M200 and Y250-M281) are covalently labelled from outside by two non-permeant specific ligands, azido-atractylate and azido-ADP. Loop C of UCP is also photolabelled by 2-azido-ADP [40]. Loop A of PiC reacts with the non-permeant eosin-maleimide [41]. It is then possible that some of the large matricial loops A, B or C might be inserted into the lipid bilayer as hairpin structures being then exposed to externally added non-permeant reactants. It has been speculated that these structures could participate in the conformational changes occuring in the transport process.

Several experimental data are in favor of multimeric state of mitochondrial carriers. Binding stoichiometry of inhibitors and adenine nucleotides to the AAC were interpreted as indications of mitochondrial carriers being functional dimers [19], whereas binding experiments with adenine-nucleotide analogues were interpreted in favor of a functional tetramer [20, 42]. Cross-linking studies of AAC [43], UCP [44] and OGC [45] support functional dimers. Numerical analysis of complex kinetic and oxoglutarate binding curves of the OGC from rat-heart has led to an oligomeric model [16]. Two substrate kinetic study of AAC has shown that the carrier is a dimeric association (Sluse *et al.*, in preparation, and [46]). The putative dimeric functional unit of MCF members presenting 6 + 6 α-helices should be related to the 12 transmembrane helices of most of the

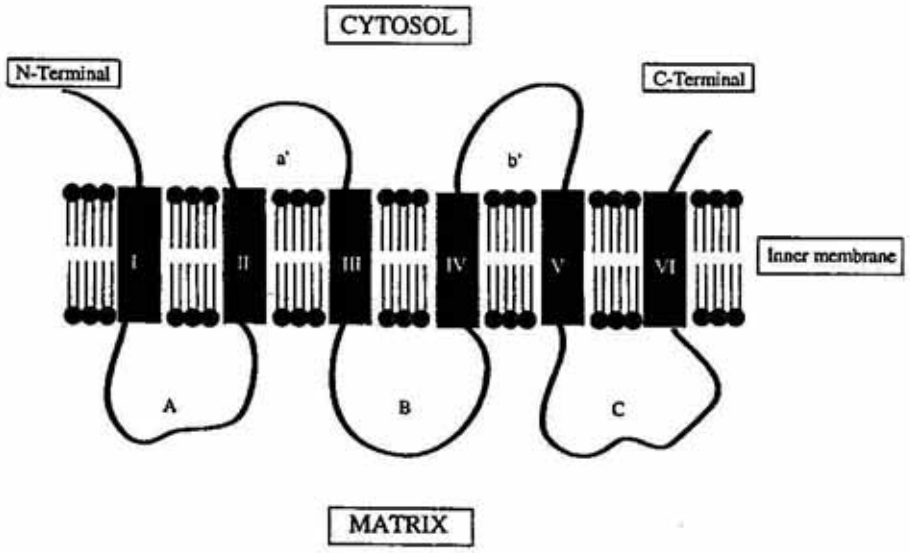

Fig. 4. Topological model of a mitochondrial carrier: the six transmembrane α-helices are numbered I to VI, the three large loops A, B and C are on the matricial side and the two short loops a' and b' are on the cytosolic side with the N- and C-termini.

prokaryotic and eukaryotic membrane carriers [47]. It must be noticed that an oxoglutarate/malate transporter containing 12-helix motif has been identified in spinach chloroplast membrane [48].

Alignement of the sequences shows that only a very few amino acids are highly conserved in MCF: mainly proline residues and acidic residues (D/E) at the end of helices I, III, V and one glycine at the begining of helices 2, 4, 6. A degenerate signature sequence proposed by Bairoch [49] is general for all members of the MCF. It is situated at the end of the first helices in the repeats: P-x[D/E]-x-[LIVAT]-[KR]-x-[RLH]-[LIVMFY]. In members of the mitochondrial carrier family, this consensus pattern is usually found twice but sometimes only once. This motif together with the tripartite structure is useful for the recognition of new members of the MCF with unknown biochemical function (as example [50, 51]). The most conserved residues are situated on the matricial side at the beginning and at the end of loops A, B, C. Despite a very little knowledge regarding the specific function of the conserved residues they could have important structural roles related to structural and functional similarities in the carrier protein family.

Using the progressive alignement TREE program of Feng and Doolittle [52], relative evolutionary distances and a phylogenetic tree can be determined. Analysis of the sequences of carriers of known and unknown functions has led to a tree with 12 branches [6, 53] representing 12 subgroups or clusters. Five clusters are proteins of known functions (AAC, UNC, PiC, OGC, CIC), seven clusters are putative carriers from various sources. It can be surprising that so many different carrier proteins have not yet been functionally identified even if several of them could be the well functionally defined major carriers.

More specific analysis can be realized within a cluster, as the AAC sub-group, and it shows that more than hundred residues are absolutely conserved [54]. It is useless guidance for site specific mutagenesis. Nevertheless, a rational strategy has been developed by Nelson [54] that has allowed the first site-directed mutagenesis of a mitochondrial carrier in yeast. A very striking peculiarity of all AAC is the presence of the RRRMMM series in the beginning of the matricial C loop. As the negative charges of adenine nucleotides are likely to interact with positive charges on the translocator during transport process, the highly conserved positive residues are candidates for such an interaction (R252, R253, R254, K38, R96, R204, R294). Moreover, it was already known that R96 is the site of a natural mutation of the AAC 2 isoform rendering yeast unable to grow on non-fermentable glycerol [55]. Several cystein residues were also mutated (C73, C244, C271) because chemical modification of AAC by *N*-ethylmaleimide has suggested that these cysteines are involved in transport activity [56–58]. The lysine 179 and 182, labelled with azido-ADP and ATP and perhaps participating in nucleotide binding, were also selected for mutation. Moreover, several other residues were mutated as tryptophane 235 and the 100% conserved proline 247. Single mutation of all seven charged amino acids results in the unability of yeast cells to grow on glycerol and lead to the biosynthesis of AAC proteins with very poor activity if any. These residues are then essential. The other residues are mutated without affecting growth on glycerol. These results indicate, at least, that the presence of the arginine triplet is crucial for transport activity as well as the four charged residues in the membrane spanners.

The non-functional mutants of the matricial arginine triplet R252-253-254 have been used by Nelson to select intragenic spontaneous suppressors [59, 60]. All the analyzed revertants (frequency 1 over 10^9) were single point mutations at a site different than the arginine triplet. Unexpectedly, revertant mutations, except 1, affect 13 different residues in a sector of the carrier near the cytosolic surface of the membrane, then on the opposite side. These residues are proposed to form a critical region that seems to be physically altered by the inactivating mutation [59]. Thus, mutations in the matrix arginine triplet could propagate a long distance structural change to the other side of the membrane. Site-directed mutagenesis studies have also been performed with PiC in yeast showing that 2 acidic residues (Glu 126-137) and one histidine are essential for function [61].

Over-expression of mitochondrial carriers in bacteria has been accomplished recently for PiC, OGC, AAC [62] and CIC [63]. They are accumulated as insoluble inclusion bodies in

Table 3
Emerging information on carrier family

Properties of carriers	Approaches		
	Kinetic	Molecular	Genetic
Binding sites on both sides of the membrane	yes	yes	–
Simultaneous exchange	yes	–	–
Native carrier may be oligomeric	yes	yes	–
Long distance interactions (internal-external)	yes	–	yes
Substrate-interacting sites into the channel	yes	–	yes

E. coli and may be solubilized and reconstituted into liposomes. The transport properties of the reconstituted OGC, for example, were shown to be the same as those determined for the native OGC.

The main information obtained until now from the three types of approaches can be summarized as followed:

- Kinetic studies have shown (1) that most of the mitochondrial carriers display binding sites simultaneously on both sides of the membrane and that a ternary complex (internal substrate — external substrate — carrier) is compulsory; (2) that binding of the substrates induces conformational changes; (3) that the catalytic step begins with a transfer of the two substrates on sites situated into a channel; (4) that active carrier may be oligomeric; (5) that strong cooperativity in binding may exist (or not) between internal and external sites as well as between one-side sites.
- Molecular studies have shown (1) that carriers may exist as dimer or tetramer, with binding sites on both sides of the membrane; (2) that the monomer unit of carriers have a similar apparent mass; (3) that topology exibits a membraneous part, an at least six-helices channel, between two extramembraneous and hydrophilic parts that may be implied in ligand bindings; (4) that a very few residues are totally conserved in the whole MCF but that inside a cluster a large number of amino acids are 100% conserved; (5) that peculiarity in a cluster may be a useful guidance for site-specific mutagenesis studies.
- Genetic studies have shown (1) that mutagenesis can be used in such a cell that can survive without the active carrier; (2) that single mutation of a residue may result in biosynthesis of carrier without activity; (3) that spontaneous revertants may appear that are single point mutations; (4) that location of revertant defines critical region in the carrier that represents a residue's sphere of influence; (5) that this region may be on the other side of the membrane; thus, mutation on one side of the membrane can propagate a structural change to the other side of the membrane; (6) overexpression of mitochondrial carriers has been achieved in bacteria; (7) genomic sequencing brings a raising number of putative carriers with unknown function.

It is obvious that kinetic studies will never bring chemical interaction information and conversely three-dimensional structure alone will never bring mechanistic information. It is also obvious that mutational analysis will significantly enrich the knowledge at the level of a molecular mechanism, only if both, the ternary structure and the kinetic mechanism are known. Nevertheless, convergent information already emerge from the three main approaches (Table 3).

As a conclusion of this overview of the MCF field, it may be asserted that in order to reach the ultimate aim in carrier research, the future challenges are definitely crystallisation, site-directed mutagenesis and elucidation of the function of the new putative carriers.

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