Minireview

The role and structure of mitochondrial carriers

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Abstract Members of the mitochondrial carrier family transport compounds over the inner mitochondrial membrane to link the biochemical pathways in the cytosol with those in the mitochondrial matrix. X-ray crystallography has recently provided us with the first atomic model of the bovine ADP/ATP carrier, which is a member of this family. The structure explains the typical three-fold sequence repeats and signature motif of mitochondrial carriers. However, the carrier was crystallised as a monomer in detergent, which is inconsistent with the consensus that mitochondrial carriers exist as homo-dimers. The projection structure of the yeast ADP/ATP carrier by electron crystallography shows that carriers could form homo-dimers in the membrane.

Key words: Mitochondrial carrier; Electron crystallography; X-ray crystallography; Structure; Membrane protein

1. Introduction

Energy-generating pathways such as the citric acid cycle and fatty acid β-oxidation are carried out inside mitochondria to couple the production of NADH to oxidative phosphorylation. Mitochondria are also required for the synthesis and degradation of amino acids (urea cycle), the synthesis of iron–sulphur clusters and haem, and the generation of heat by dissipation of the proton gradient. In addition, mitochondria replicate mitochondrial DNA, and transcribe and translate mitochondrial mRNA. To carry out these pathways, solutes have to be continuously exchanged between the mitochondrial matrix and the cytoplasm. Members of the mitochondrial carrier family facilitate many of the transport steps over the inner mitochondrial membrane.

When the first amino acid sequences of mitochondrial carriers became available [1–4], it was clear that the carriers form a well-defined family [5,6]. One defining feature of the carriers is the so-called tripartite structure, consisting of three homologous sequence repeats of about 100 amino acid residues each, which was first noted in the published sequence of the bovine ADP/ATP carrier [6] (Fig. 1). The carriers also have a signature motif, which contains the P-X-[D/E]-X-X-[R/K] sequence and is conserved in all members of the family and in all three repeats (Prosite PS50920) (Fig. 1). The general consensus in the field is that the carriers are functional as homodimers based on size exclusion chromatography [7–9], equilibrium sedimentation [7,8], neutron scattering [10], native gel electrophoresis [9] and experiments with cross-linkers [11,12] or tagged monomers [13].

The typical three-fold sequence repeats and signature motif of mitochondrial carriers make it relatively easy to retrieve sequences of mitochondrial carriers from genome databases. Mitochondria of eukaryotes contain typically between 35 and 55 different carriers, which are all encoded by genomic DNA.

Not all members of the mitochondrial carrier family are actually targeted to mitochondria. An adenine nucleotide carrier has been characterised in yeast peroxisomes [14] and an ADP-glucose carrier in amyloplasts, which are modified chloroplasts involved in starch storage [15]. An ADP/ATP carrier was also identified in hydrogenosomes, which are organelles that carry out anaerobic energy metabolism using protons as terminal electron acceptors to produce hydrogen [16]. However, no homologues of mitochondrial carriers have ever been found in prokaryotes or archaea, meaning that they are a uniquely eukaryotic invention.

2. The role of mitochondrial carriers in cellular metabolism

The human genome likely encodes 48 different mitochondrial carriers. If one assumes that transporters with very high sequence identity have similar functions then it is estimated that there are 39 possible functions in total, of which approximately a third have now been assigned.

Several strategies have been applied for the identification of carriers. Carriers have been over-expressed in inclusion bodies in *Escherichia coli*, purified and reconstituted into liposomes for characterisation by transport assays [17]. Another approach uses the over-expression of carriers in yeast mitochondria, followed by purification and reconstitution (e.g. [18]). An alternative method was recently developed using the nisin expression system of the Gram-positive *Lactococcus lactis* [19]. The advantage of this system is that the carrier can often be directly characterised in whole cells or membrane vesicles without the need for purification and reconstitution. Finally, the development of genetic techniques has allowed the construction of specific deletion strains or gene knock-outs. The physiological characterisation of isolated mitochondria can be a good strategy to obtain clues about the possible function of the carrier, but since the deletion can cause rather complicated add-on effects the substrate specificity can never be assigned with confidence in that way.

In the following sections the involvement of mitochondrial

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carriers in mitochondrial DNA replication, metabolic energy generation and amino acid metabolism will be discussed, focussing mainly on the mammalian mitochondrion. Information will be used that was obtained for yeast and plant mitochondrial carriers to indicate where possibly essential information is still missing. For details and references on the biochemical properties of the carriers and their involvement in diseases the reader is referred to a recent review by Palmieri [20].

2.1. Metabolic energy generation

The degradation of sugars by glycolysis in the cytosol yields pyruvate as the final product (Fig. 2). Pyruvate is transported into the mitochondrion for the conversion to acetyl-CoA. The human pyruvate transporter has not been identified, but the yeast carrier was reported to be encoded by gene YIL006w [21]. The assignment is based on the characterisation of pyruvate transport in mitochondria isolated from deletion strains, but needs to be confirmed by using purified protein in a reconstituted system. For conversion of pyruvate to acetyl-CoA, the mitochondrion also requires the accumulation of coenzyme A itself. In yeast, the carrier might be encoded by YHR002w, because it was found to be essential for the maintenance of coenzyme A levels [22]. The human isoform, known as the Graves' disease carrier, complements the phenotype in yeast, but the substrate specificity of these carriers has not been determined directly. The conversion of pyruvate to acetyl-CoA, which is catalysed by pyruvate dehydrogenase, also requires several other co-factors like lipoamide, thiamine pyrophosphate, NAD^+ and FAD. Yeast carriers have been described for thiamine pyrophosphate [23] and FAD [24], while plants might have a transporter for NAD^+ [25], but it has not established whether it belongs to the mitochondrial carrier family.

Acetyl-CoA enters the citric acid cycle, which produces reducing equivalents and GTP (Fig. 2). Several human carriers are involved in exchanging intermediates of the citric acid cycle with the cytosol such as the dicarboxylate [26], tricarboxylate carrier [27] and oxoglutarate/malate carrier [28]. Yeast mitochondria also contain a fumarate/succinate carrier [29] and an oxaloacetate carrier [30].

The complexes of the respiratory chain generate a gradient of protons across the mitochondrial inner membrane and use reducing equivalents as electron donors (Fig. 2). The proton motive force is used by the ATP synthase to synthesise ATP from ADP and inorganic phosphate [31]. The ADP/ATP carrier catalyses the exchange of cytosolic ADP for ATP synthesised in the mitochondrial matrix and thereby replenishes the

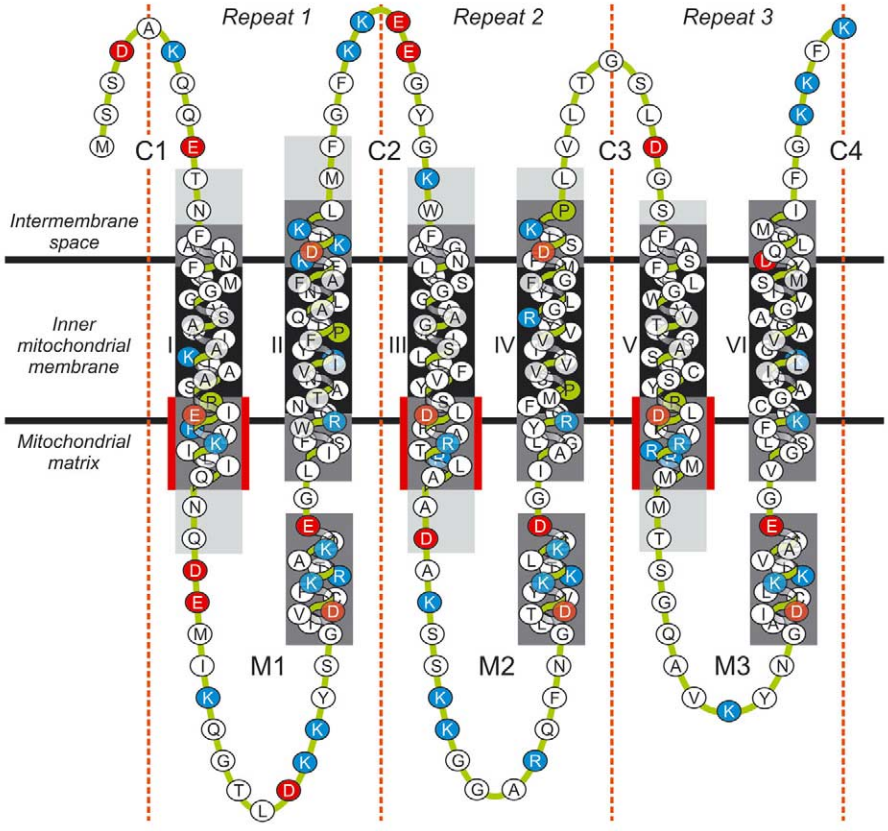

Fig. 1. Topology model of the yeast ADP/ATP carrier (AAC3). The black areas indicate the regions that were predicted to be transmembrane on the basis of hydropathy profiling. The dark grey areas were proposed extensions of the α-helices [44] based on the simple assumption that all conserved regions were likely to have α-helical structure. The light grey regions show the additional amino acid residues found in the experimentally determined α-helices based on similarity with the structure of the bovine ADP/ATP carrier [45]. Red dotted lines indicate the borders of the three sequence repeats of the tripartite structure. The amino acid identity and similarity between the repeats in AAC3 are approximately 20% and 50%. The regions with the signature motif are bordered by red bars. Residues with negative charges are indicated in red, those with positive charges in blue and proline residues in green. The loops in the mitochondrial matrix are numbered M1, M2 and M3, the cytoplasmic loops C1, C2, C3 and C4. The helices are indicated with Roman numerals on the left hand side of the helices.

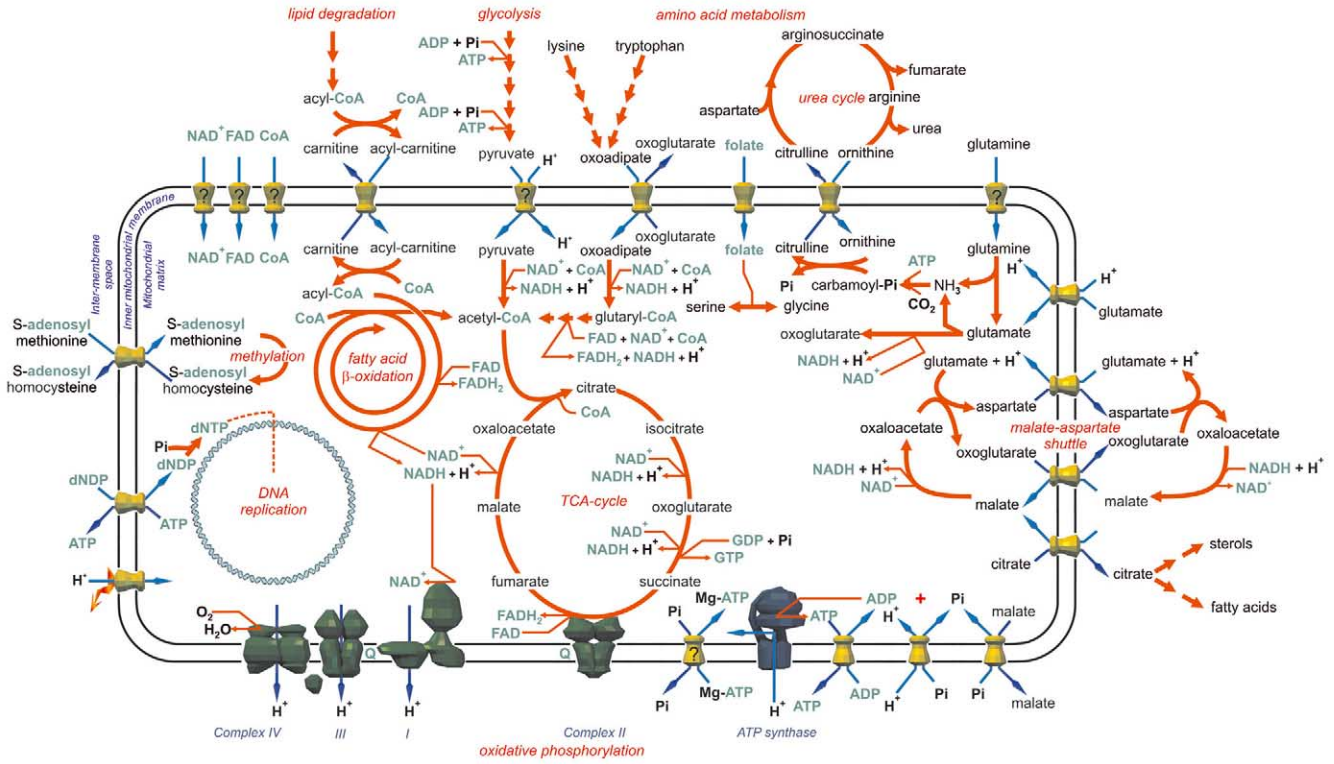

Fig. 2. Role of mitochondrial carriers in mitochondrial and cellular metabolism. A schematic representation of the inner membrane of a mammalian mitochondrion with the ATP synthase, the complexes of the respiratory chain and mitochondrial carriers. The permeable outer mitochondrial membrane is not shown. Red arrows indicate the metabolic pathways, which are simplified to emphasise key metabolites. Co-factors and high-energy intermediates are indicated in green. Note that the same metabolites appear at different sites, meaning that in principle the corresponding pathways are linked. Blue arrows indicate the directionality of the transport steps over the inner mitochondrial membrane as carried out by the membrane proteins. The human transporters of CoA, glutamine, FAD, NAD^+, and pyruvate are marked with a question mark, because it is currently unknown whether they belong to the mitochondrial carrier family.

eukaryotic cell with metabolic energy [32]. The adenine nucleotide exchange is strictly equimolar, meaning that the total adenine nucleotide pool in the matrix will not change as a consequence [32]. When the mitochondrion divides during cell division the total adenine nucleotide content would halve and the mitochondrion therefore needs a carrier to replenish the adenine nucleotide pools. The ATP-Mg/Pi carrier could fulfil this role, but the gene encoding the carrier has not been identified to confirm it belongs to the family [33]. The dicarboxylate [26] and phosphate carriers [3] are important for the maintenance of inorganic phosphate levels in the mitochondrial matrix.

Mitochondria also play a central role in the oxidation of fatty acids derived from lipid breakdown, which is a very efficient source of metabolic energy (Fig. 2). Fatty acid chains are transferred from coenzyme A to carnitine by carnitine palmitoyltransferase in the outer mitochondrial membrane. The carnitine/acyl-carnitine carrier transports acyl-carnitine into the mitochondrial matrix in exchange for free carnitine, which is generated when the acyl chains are transferred back to coenzyme A [34]. Acyl-CoA then enters fatty acid β-oxidation for the cyclical breakdown of the carbon chain. In each cycle, $FADH_2$ and NADH are produced as well as acetyl-CoA by removal of two carbon atoms from the chain. Acetyl-CoA enters the citric acid cycle for the production of additional reducing equivalents, which are all used in oxidative phosphorylation for the production of ATP. An important head group of lipids, choline, is also transported into mitochondria, where it is oxidised to betaine by an NAD-linked enzyme [35], but it is unknown whether this transport step is carried out by a mitochondrial carrier. Finally, the proton gradients generated by the electron chain can be dissipated by the uncoupling protein to produce heat [36].

2.2. Amino acid metabolism

Mitochondria are also important in the synthesis and degradation of amino acids (Fig. 2). Ammonium, which is derived from the conversion of glutamate to oxo-glutarate or glutamine to glutamate, is fixed as carbamoyl-phosphate in the mitochondrial matrix. Carbamoyl-phosphate reacts with ornithine to form citrulline, which is transported out of mitochondria by the citrulline/ornithine exchanger. Citrulline enters the urea cycle, which leads to the production of urea and ornithine as the final products. Ornithine is used as a substrate in the exchange for citrulline [37]. The urea cycle is also important for the metabolism of arginine, aspartate and asparagine (Fig. 2).

Glutamine is transported into the mitochondrial matrix by the glutamine transporter, but the corresponding gene has not been identified meaning that it might not be a member of the family. Glutamate is transported into the mitochondrion by the glutamate transporter [38] and the aspartate/glutamate exchanger [39]. The aspartate/glutamate exchanger [39] and the oxoglutarate/malate carrier [28] are also part of the malate–aspartate shuttle that is used for the removal of reducing equivalents from the cytosol by a series of conversions and

transport steps (Fig. 2). The transport of glutamate could also play a central role in the metabolism of proline and histidine.

Lysine and tryptophan are broken down to oxoadipate, which is transported to the mitochondrial matrix in exchange for oxoglutarate [40]. Oxoadipate is broken down to two acetyl-CoAs with the production of reducing equivalents. Acetyl-CoA enters the citric acid cycle, which leads to the production of oxoglutarate, the other substrate in the transport exchange.

Alanine, serine and cysteine are degraded in the cytosol to pyruvate, which enters the mitochondrion via the pyruvate carrier, while phenylalanine and tyrosine are broken down to malate, which is transported by the dicarboxylate carrier [26]. The human transporter for folate, which is used for interconversion of glycine and serine in the mitochondrial matrix, has been described and is a member of the mitochondrial carrier family [41].

2.3. Mitochondrial DNA replication

Mitochondria have their own DNA, which encodes several proteins of the electron transport chain complexes and ATP synthase. Mitochondria have a deoxynucleotide carrier that could supply mitochondria with deoxynucleotides for mitochondrial DNA replication [42]. For methylation of DNA, RNA and proteins, mitochondria use *S*-adenosylmethionine, which is translocated by a specific transporter of the mitochondrial carrier family [43].

3. The structure of mitochondrial carriers

In 2003, the projection structure of the yeast ADP/ATP carrier [44] and the atomic model of the bovine ADP/ATP carrier were published [45]. Both efforts concentrated on the ADP/ATP carrier, because the carrier is a good target for structural work. First, the carrier is one of the best studied and characterised members of the mitochondrial carrier family (reviewed in [46–48]). Second, the carrier is usually the most abundant transporter in mitochondria, which aids the isolation and purification of starting material for crystallisation trials. Third, the carrier can be inhibited in two distinct conformational states, which are reasonably stable in detergents. The carrier binds carboxy-atractyloside or atractyloside in the cytoplasmic or *c*-state and bongkrekic acid in the matrix or *m*-state (reviewed in [47,48]).

The projection structure was obtained using only a few milligrams of over-expressed yeast ADP/ATP carrier (AAC3) [44]. The carrier was inhibited by atractyloside, purified in dodecyl-maltoside and reconstituted into two-dimensional crystals. Images of frozen hydrated crystals were recorded by electron cryo-microscopy and the projection structure was calculated to 8 Å resolution. It was clear from the projection structure that the AAC3 molecule had pseudo three-fold symmetry in agreement with the three-fold sequence repeats that are typical of members of the mitochondrial carrier family (Fig. 3A). The projection structure also showed that the carrier was most likely a bundle of six transmembrane α-helices rather than an integrated bundle of 12. This was a surprising finding in view of the general consensus that mitochondrial carriers are homo-dimers in structure and function [7–13]. This meant that dimer formation could only occur via association of two structurally separate monomers. Interestingly, the two-dimensional crystals contained homo-dimers, which seem to be the main crystallising unit in two different crystal forms. The pore in the centre of the monomer was clearly the most likely site for translocation of adenine nucleotides [44].

The predicted α-helices based on the hydropathy profiling alone would be too short to form a bundle of six tilted α-helices. Therefore, a new topology model was proposed based on the simple assumption that well-conserved regions in mitochondrial carriers were likely to form secondary structure (Fig. 1). Two predictions were made: (i) the region of the signature motif would be part of the odd-numbered transmembrane α-helices and (ii) short α-helices would be present in the matrix loops at the N-terminal side of the extended even transmembrane α-helices (Fig. 1) [44].

Many of these issues were entirely resolved by the atomic structure of the bovine ADP/ATP carrier, which was solved to 2.2 Å [45]. The ADP/ATP carrier is the most abundant transporter in bovine heart mitochondria and was purified in the detergent lauryl-amidodimethyl-propylaminoxide (references in [45]), which was first used by Klingenberg for this purpose [49]. Key to success of the crystallisations was the removal of excess detergent by BioBeads in a controlled manner [50]. The carrier was crystallised in complex with the inhibitor carboxy-atractyloside, which locks the carrier in the cytoplasmic state like atractyloside [45].

The structure explains two general sequence features of mitochondrial carriers in molecular detail: the three internal sequence repeats and the conservation of the signature motif. The main structural fold is indeed a six α-helical bundle with pseudo three-fold symmetry (Fig. 3B,C). The fold of the three repeats in the structure is very similar with root-mean-square deviations of less than 2 Å [45]. The prolines of the signature motif induce a sharp kink in the odd-numbered α-helices, which close the central pore of the α-helical bundle on the matrix side (Fig. 3B,C). These particular helices are held together by salt bridges and hydrogen bonds, which are formed by the charged residues of the three signature motifs (Fig. 3C) [45]. The existence of charge pair networks had been predicted through the analysis of second site revertants in yeast [51]. It is possible to see how a rearrangement of these bonds might cause opening of the carrier on the matrix side when the molecule changes conformation. The structure also provides detailed information on how carboxy-atractyloside is bound in the open cavity that is formed by the bundle [45]. Interestingly, residues involved in binding of carboxy-atractyloside are also involved in the charged pair network, suggesting that substrate binding and opening of the central pore might be related events.

All features of the projection map of the yeast ADP/ATP carrier can be explained by the structure of the bovine ADP/ATP carrier. The asymmetric feature in the core of the molecule can now be attributed to atractyloside. The surprising finding of the X-ray structure was the absence of dimers in the crystals, which cannot be easily explained in view of earlier observations [7–13]. Using the bovine structure we are carrying out a detailed analysis of the crystal packing in the membrane to study the interface of the putative dimer seen in the projection structure.

So what more needs to be done to advance our understanding of the transport cycle in structural terms? The inhibitors that were used in both studies are not structural analogues of adenine nucleotides, so we still do not know where exactly ADP binds. However, the inhibitors do prevent ADP binding,

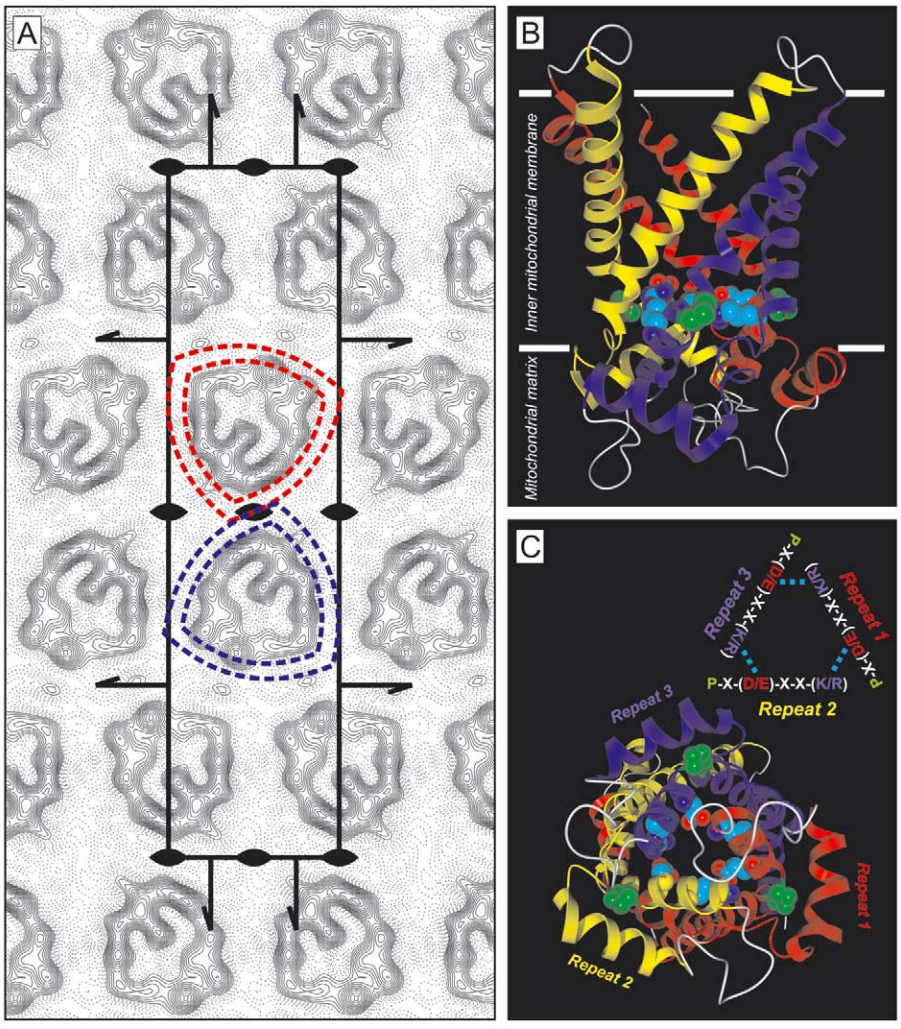

Fig. 3. Projection map of the yeast ADP/ATP carrier and atomic model of the bovine ADP/ATP carrier. A: The projection map of yeast AAC3 with atractyloside bound, which was calculated from merged amplitudes and phases of five independent lattices with $p22_12_1$ symmetry imposed [44]. The unit cell is displayed with the *a*-axis (42.5 Å) horizontal and the *b*-axis (172.1 Å) vertical. Solid lines indicate density above the mean, while negative contours are shown as dotted lines. At this density cut-off, side chain densities are not shown. The bold dotted lines in the equilateral triangles indicate the approximate area that the side chains would occupy. The two molecules of the putative homo-dimer are shown in blue and red. B,C: Membrane (B) and matrix (C) view of the bovine ADP/ATP carrier model with carboxy-atractyloside bound, based on the coordinates provided by Pebay-Peyroula and colleagues (1okc) [45]. Only the secondary structure elements are shown and the carboxy-atractyloside molecule is omitted. The conserved elements of the first repeat are shown in red, of the second in yellow and of the third in blue. The white bars indicate the approximate position of the inner mitochondrial membrane. The atoms of the proline residues of the signature motif are depicted in green spheres and the salt bridge residues in CPK coloured spheres. The models are shown in perspective. Also shown is the schematic representation of the charged pair relay by the signature motif of the ADP/ATP carrier. The figure was made with Yasara.

indicating that the binding sites may partially overlap. Mutagenesis has identified several residues important for the binding of adenine nucleotides and some of these are indeed involved in the binding of carboxy-atractyloside too [45]. This, however, means that the inhibited state of the carrier may not resemble the empty cytoplasmic state of the carrier, because the inhibitor is partially bound to the binding site. It may also not resemble the nucleotide binding state either, because the binding of ADP must lead to a change in conformation in the transport cycle, which is clearly prevented in the inhibited state. How ATP binds to the matrix state of the carrier is of course not known at all. The problem for structural work is that the uninhibited states are quite unstable in detergent. The use of structural analogues of adenine nucleotides and/or the selective mutagenesis of the putative binding sites could be a way to obtain stable intermediates for structure determination.

The structure does not provide insight into the dynamic changes that need to occur when the transporter changes from cytosolic to matrix state either. Therefore, another important target of structural work is the bongkrekate-inhibited state, which may resemble the carrier with the binding site open to the matrix side, but is in effect also an artificial state. The transition state between the two conformations is also intriguing. It is very unlikely that the carrier transforms into a channel-like conformation as suggested [45], since this may lead to the influx of protons during translocation.

What about the oligomeric state of the carrier and its relation to conformational changes? It is clear that mitochondrial carriers are structurally monomers in the membrane [44] and

in detergent [45], but they could associate to form a dimer as suggested [7–13]. One important implication of the homo-dimer model is that the two halves could coordinate the change in conformation in a cooperative manner to ensure the equimolar exchange of adenine nucleotides [52]. This means that the intact homo-dimer needs to be crystallised with the two associated monomers in different conformational states of the transport cycle. It is clear that there is still a considerable amount of work to do before we understand how these transporters work in structural detail.

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