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# Structure-function relationships of the ADP/ATP carrier

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Mitochondria contain a multitude of solute carriers which deal with the metabolite exchange between the inner- and extramitochondrial space [1–3]. These carriers form a gene family as based on the amino acid sequences. Starting with the primary sequence of the ADP/ATP carrier (AAC) [4], the sequence of the uncoupling protein (UCP) [5] was found to be similar. This was followed by the amino acid sequence of the phosphate carrier which firmly established the existence of a mitochondrial carrier family [6]. Subsequently, this was extended when the structures of the ketoglutarate-malate carrier [7] and the citrate-malate carrier became known [8]. In the meantime, several sequences of unknown function in mitochondria and even in other organelles have been found with a similar structural motive as the mitochondrial carrier family. For this reason the mitochondrial carrier family is part of a 'superfamily' [9].

These carriers are distinguished by the simplicity of their structures comprising only about 300 amino acids [10]. An internal 3-fold symmetry can be deduced from the 3-fold similar domains of about 100 residues each. These features have no similarity in any other transport protein known. The carriers form structurally and functionally homodimers which also is quite unique among carriers.

Physical data on the structure and space are only known from hydrodynamic data for the AAC and for the UCP. Particularly for the AAC a protein detergent micelle established an oblate and elongated ellipsoid shape in which the homodimer is located on the short axis with the twofold symmetry axis [11]. Thus, a high percentage of the protein mass is encased by the detergent, which should correspond to the phospholipid boundary layer in the membrane. Another physical parameter is the content of $\alpha$-helical structure established by CD measurements. It is bound both in the AAC and UCP to about 41%. The folding through the membrane has been predicted first on the basis of the primary structure. The signal to noise ratio in hydrophobicity plots is rather poor due to a high content of hydrophilic groups. But the s/n is considerably increased by postulating that the three repeats have a similar internal structure with each containing a pair of transmembrane helices amounting to a total of six transmembrane helices. A further decrease of the s/n in hydrophobicity is gained from the similarity of different carriers, again supporting the six transmembrane helical model. This agrees also with the 40% $\alpha$-helical content. This arrangement also incorporates the localization of both the N and C terminus to the cytosolic site.

In this model (Fig. 1) three, about 40 residues long, hydrophilic sections are located between the two transmembrane helices in each domain containing numerous charged residues [12,13]. Probing the distribution of lysine in the AAC with the membrane impermeant lysine reagent pyridoxalphosphate, however, gave results which are not completely in line with the six-transmembrane-helical model. Furthermore, it was shown with various covalently binding ATP derivatives that these were incorporated in the AAC in the hydrophobic section of the second domain and in UCP in domain three, although these nucleotides are not transported and act only from the cytosolic site. These data suggested to us that in each domain a loop extends into the membrane from the hydrophilic matrix section and that these three hydrophilic loops line the

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translocation path. They are visualized to be surrounded by the six helices [12,13]. More recently non-helical loops, possibly β-hairpins, are postulated to exist also in the channels for $K^+$, $Na^+$ and $Ca^{2+}$, again surrounding the actual ion channel [14].

For elucidating the structure-function relationships in the AAC, we have concentrated in recent years on site-directed mutagenesis of the AAC2 in yeast. The candidate groups selected for mutations were those which from the previous studies were assumed to have an essential function in the transport. Another criterion was to select groups which in the proposed structural model occupied conspicuous positions (Fig. 1).

The first group encompassed the cysteines, at least one of these were proposed to be essential for transport [15]. A second group of mutations was directed at three arginines strikingly located in transmembrane helices. In each domain the second helices contained such an arginine. These arginines are conserved not only in all AACs known but also in other carriers. Thirdly, the arginine triplet on the matrix site of the third domain was mutated because of its striking composition and complete conservation in all AACs. In other carriers at the same position instead of the triplet a positive doublet is conserved with the middle charge replaced by a neutral residue. The fourth group covered several charged residues of functional or structural importance, i.e., the only intrahelical lysine K38, and lysines in the hydrophobic section of the second domain, K179 and K182. These we might place on the tip of the intrahelical loop and have been found in bovine heart mitochondria to react with pyridoxalphosphate in dependence on the functional state of the AAC. Furthermore, one negatively charged residue D149 was selected out of those which strikingly delimit the transmembrane helices in a highly conserved manner in all mitochondrial carriers.

The results of the mutations were followed on four different levels, on the growth characteristics of the cells, on the level of mitochondria, the isolated protein and the reconstituted proteoliposomes. Growth on a non-fermentative source was a criterion for the functionality of the carrier. In the isolated mitochondria the content of carriers was assayed by immunoblots, binding of the inhibitors [$^3$H]CAT or [$^3$H]BKA. The respiratory capacity, the cytochrome content of the mitochondria was measured and the rate of ATP synthesis was determined also as a measure of the ADP/ATP transport capacity based on the fact that most of the ATP synthesis rate was sensitive to the inhibitors of AAC transport. The AAC was isolated and reconstituted for the transport assay.

Mutations of the cysteine C73, C244, C277 to serine proved to be not damaging for the transport activity. This is in line with the experience of mutations at other carriers in which so called essential cysteines could be replaced by serine or other amino acids without apparent transport activities. Obviously, the substitution at the SH-group of an alkylating reagent has either an obstructing effect on the translocation or disturbs some hydrogen bond. The reconstitution of C73S, however, required addition of cardiolipin to the phospholipids, whereas the wild-type activity does not require cardiolipin (Table 1). There was an absolute requirement for cardiolipin which could also not be replaced by any other acidic phospholipids. Detailed studies further

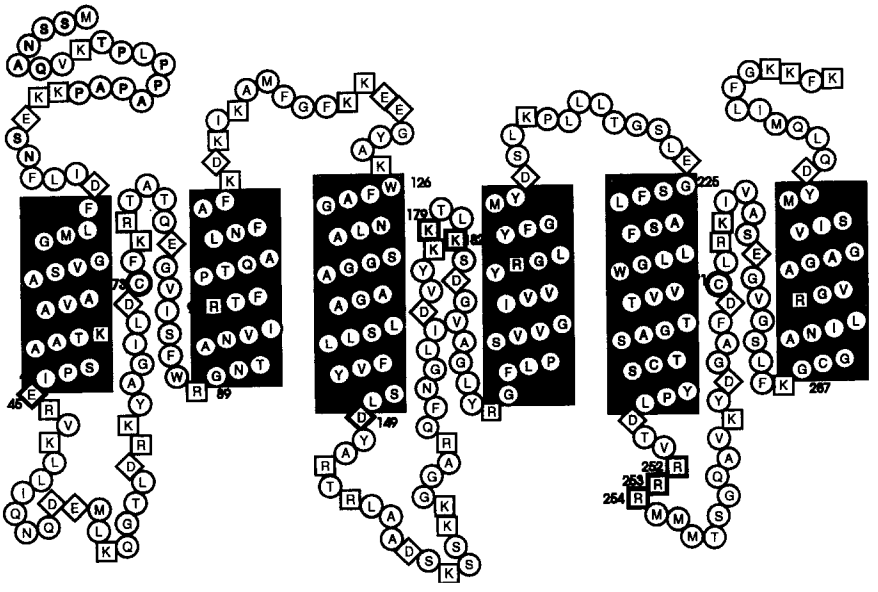

Fig. 1. The secondary structure of the ADP/ATP carrier. The example represents the AAC2 for *Saccharomyces cerevisiae*. The polar residues are symbolized as follows: acid ◇, basic □. The mutated residues are represented by bold frames.

Table 1
Cardiolipin dependence of ADP/ATP exchange activity

| Mutant | Activity (μmol/min per g protein) | |
|---|---|---|
| | −CL | +CL [a] |
| Wild | 1050 | 1100 |
| C73S | 5 | 290 |
| C244S | 900 | 1050 |
| C271S | 420 | 600 |
| K179I | 10 | 350 |
| K179I +K182I | 10 | 200 |
| R96H | 120 | 130 |
| R294A | 600 | 630 |

[a] CL = cardiolipin 3 to 6% w/w PC.

showed that this was due to loss of bound cardiolipin on isolation of the C73S-AAC, whereas the isolated wild-AAC retained 6 mol cardiolipin [15].

The three intrahelical repeat arginines proved to be most interesting. R96 was found simultaneously to be the site of mutation in the op1 (pet9) mutant [16,17]. All these mutations led to a glycerol negative growth (Tables 2 and 3). The ATP synthesis rate in mitochondria proved to be only a few percent as compared to the wild-type. As to be expected, the respiratory capacity is also decreased in these mutants, however to a lesser extent than the oxidative phosphorylation. The ATP synthesis rate is also compared with the content of the AAC in mitochondria as measured by [$^3$H]CAT binding. Here it becomes clear that in the gly$^-$ mutants the expression of the AAC as measured by the [$^3$H]CAT binding is diminished but not as much as the ATP synthesis, when compared to the wild type. This is best illustrated by the ratio of the ATP synthesis rate to [$^3$H]CAT binding (Table 3). In general, this ratio is

Table 2

| Mutant | Growth on glycerol | Reconstituted AAC activity [a] (μmol/min per g prot.) |
|---|---|---|
| Wild | + | 1100 |
| C73S | + | 300 |
| C244S | + | 1000 |
| C271S | + | 600 |
| R96H | − | 150 |
| R204L | − | 0 |
| R294A | − | 800 |
| R252I | − | 10 |
| R2531 | − | 20 |
| R2541 | (+) | 30 |
| K38A | − | 0 |
| K179M | + | 400 |
| K179I +K182I | + | 300 |

[a] Exchange ADP → ADP.

Table 3
ATP-synthesis rate [a] in mitochondria

| Mutant | $V_{ATP}$ (μmol/min/g prot.) | $V_{ATP}$ | |
|---|---|---|---|
| | | [$^3$H]CAT-binding (1/min) | Respiration |
| WT | 124 | 240 | 480 |
| R 96 H | 3.8 | 15.2 | 44 |
| R204 L | 0 | – | 0 |
| R294 A | 14 | 26.7 | 91 |
| R252 I | 0.5 | 6.8 | 10 |
| R253 I | 0.2 | 3.2 | 3 |
| R254 I | 0.8 | 11.7 | 19 |
| K 38 A | 0.8 | 2.9 | 20 |
| K179 M | 75 | 167 | – |
| D 149 S | 0.5 | 4.7 | – |
| E 45 G | 46 | 136 | |

[a] CAT and BKA-sensitive portion. CAT binding should represent the content of AAC. Thus $V_{ATP}$/[$^3$H]CAT binding gives the turnover number of the AAC.

only a few percent of the wild-type activity, only in the R96H and particularly R294A it amounts to 9 or even 18% of wild-type activity. Possibly, these capacities measured in vitro may be lower in the cell because of different ADP/ATP ratios, since even these two mutants do not support growth on glycerol. Elimination of each of the arginines in the R252–254 triplet nearly completely prevents ATP synthesis in mitochondria. In this case also the [$^3$H]CAT binding is very low.

On reconstitution of the isolated AAC from these mutants the transport activities reveal some surprises. The basic transport activity of ADP/ADP exchange is still relatively high in R294A (70%) and surpasses 12% of wild-type activity in the R96H mutant, although both mutants are gly$^-$. However, if we measure the transport rate of ATP the picture changes drastically. In the wild type the ATP/ADP (T/D) exchange is even higher than the basic ADP/ADP (D/D) exchange rate when the electrical charge differences are compensated by the movement of valinomycin plus $K^+$(Table 4). In contrast, in the R294A mutant the T/D rate is much lower, only about 12 to 15% of the D/D rate. Similar relations are found for the R96H mutant AAC. Thus the intrahelical R294A mutation lowers specifically the transport of ATP versus ADP. This has important implications. The absolute T/D rate would still be sufficient for growth on glycerol but the competition of the D/D mode with the T/D mode would still further lower the ATP export and therefore makes this mutant highly inefficient. This can be expressed in the 'efficiency' of transport which is calculated by multiplying $V_{T/D}$ with the 'competition ratio' $V_{T/D}$ ($V_{D/D} + V_{T/D}$) (Table 4). Thus, the efficient exchange range is non-linearily related to the absolute rate. The comparison with the exchange-limited ATP

Table 4
The efficiency of exchange in an intrahelical arg mutant and its revertant

| Strain | Growth on Glycerol | $V_{D/D}$ | $V_{T/D}$ | Efficiency $\frac{V^2_{T/D}}{V_{D/D}+V_{T/D}}$ | ATP synthesis $V_{ATP}$ |
|---|---|---|---|---|---|
| Wild | + | 1060 | 1380 | 495 | 124 |
| R294A | – | 800 | 180 | 21 | 14 |
| Revertant R294A + E45Q | + | 45 | 80 | 51 | 23 |

Values are expressed in μmol/min per g protein.

synthesis rate in mitochondria gives a good agreement with the efficiency in the mutant and revertant. Correspondingly, the efficiency is higher in the revertant than in the mutant, opposite to the absolute rates.

We cannot conclude that the R294A is specifically involved in the binding of the additional charge of ATP since arginines at homologous positions are also present in other carriers. We can, however, conclude that all the three intrahelical arginines are essential for the electrically active branch of the exchange, i.e., the translocation of the ATP, whereas the ADP transport is electroneutral. The deletion of the R204 effectively decreases the transport activity to zero in all modes and shows that this residue is absolutely essential for transport. The same is true for the R252–254 triplet which has very little transport activity also in the reconstituted system.

The intrahelical lysine K38A mutation also completely inactivates the AAC. On the other hand, mutations of K179 and K182 proposed to be at the intrahelical loop still retain growth on glycerol. Correspondingly, these mutants AAC in reconstitution retained about 30% of wild-type activity. More interesting with these mutants is their absolute dependence on cardiolipin (Table 1). It is suggested that the lysine in these positions might be involved in the binding of the cardiolipin head groups.

Mutation of the helix terminating D149S resulted in complete inactivation. These highly conserved acidic groups found to be in all carriers are obviously important, according to our model, in stabilizing the transmembrane helices.

In conclusion, site-directed mutations in particular of charged residues have revealed a number of unexpected functionally important structure-function relationships. Particularly intrahelical charged residues seem to be of major importance in sustaining activity and in particular to sustain the electrically active transport of ATP. The unique role of the only intrahelical lysine is shown. Also extra helical lysine clusters seem to be involved in the binding of cardiolipin head groups and thus explain the absolute and unique binding of and specificity for cardiolipin of the AAC.

Further results will be shown on suppressor mutations or revertants of gly$^-$ mutants in which functionally important charge pairs are discovered [18]. In particular for the intrahelical arginines striking results are obtained demonstrating that not so much the absolute activity of the carrier but rather the productive capacity T/D decides on the usefulness of the AAC in sustaining sufficient ATP supply by oxidative phosphorylation to the cytosol.

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