Projection Structure of the Atractyloside-inhibited Mitochondrial ADP/ATP Carrier of Saccharomyces cerevisiae*

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ADP/ATP carriers in the inner mitochondrial membrane catalyze the exchange of cytosolic ADP for ATP synthesized in the mitochondrial matrix by ATP synthase and thereby replenish the eukaryotic cell with metabolic energy. The yeast ADP/ATP carrier (AAC3) was overexpressed, inhibited by atractyloside, purified, and reconstituted into two-dimensional crystals. Images of frozen hydrated crystals were recorded by electron microscopy, and a projection structure was calculated to 8-Å resolution. The AAC3 molecule has pseudo 3-fold symmetry in agreement with the 3-fold sequence repeats that are typical of members of the mitochondrial carrier family. The density distribution is consistent with a bundle of six transmembrane α-helices with two or three short α-helical extensions closing the central pore on the matrix side. The AAC3 molecules in the crystal are arranged in symmetrical homo-dimers, but the translocation pore for adenine nucleotides lies in the center of the molecule and not along the dyad axis of the dimer.

Mitochondrial carriers are a family of integral membrane proteins that transport metabolites and co-factors across the inner membrane of the mitochondria. Yeast mitochondria contain 35 members of the mitochondrial carrier family (1), whereas human mitochondria have close to 60 different carriers. The transported substrates are required for metabolic energy-generating pathways, such as the citric acid cycle and fatty acid β-oxidation, which take place in the mitochondrial matrix. The carriers also perform crucial transport steps in amino acid synthesis and degradation and mitochondrial DNA replication. Uncoupling proteins, which generate heat by dissipation of the proton gradient, are also members of the mitochondrial carrier family (2).

Mitochondrial carriers have a tripartite structure consisting of three homologous repeats of about 100 amino acid residues (3, 4). Each repetitive domain consists of two membrane-spanning α-helices connected by a large hydrophilic loop in the matrix, whereas the repeats are linked by shorter loops. Both the amino and carboxyl terminus are located in the space between the inner and outer mitochondrial membrane (5–7). Each repeat also has a signature motif, P-X-[DB]-X-[LIVAT]-[RK]-X-[LRH]-[LVMFY]-[QGAIW], which is characteristic for mitochondrial carriers (PROSITE PS00215). Biochemical and biophysical studies have suggested that the functional form of the carriers may be a homodimer, but direct structural evidence for this notion has not been obtained (6–14).

The ADP/ATP carrier is an archetypal member of this family and exchanges cytosolic ADP for ATP produced in the matrix by the ATP synthase. The substrate specificity is restricted to adenine nucleotides and the equimolar exchange of ADP and ATP is electrogenic (15). Inhibitor studies have revealed that the ADP/ATP carrier exists in two conformational states in the mitochondrial membrane. The carrier binds carboxyatractyloside or atractyloside in the cytoplasmic or β-state and bongkrekic acid in the matrix or α-state (16).

Consistent with its central function in energy metabolism, the nuclear genome of Saccharomyces cerevisiae contains three genes coding for isoforms of the ADP/ATP carrier. The isoforms AAC1 and AAC2 are expressed under aerobic conditions, whereas AAC3 is expressed under anaerobic conditions, but all three carriers have similar enzymatic properties (17).

No structural information has been obtained for any mitochondrial carrier. Here we report the projection structure of yeast AAC3 determined by electron crystallography. The projection map shows that the molecule has pseudo 3-fold symmetry compatible with the tripartite structure predicted from the amino acid sequence (4).

**EXPERIMENTAL PROCEDURES**

**Cloning of Yeast Expression Vectors and Transformation—**The yeast aac3 gene was cloned into the pYES-Pac2-aac2 vector (18), resulting in the expression vector pYES-Pac2::aac3. An NH₂-terminal nine histidine tag and a factor Xa cleavage site were introduced at the Ncol site by kinase treatment and annealing of synthesized primers, leading to the vector pYES-Pac2::NH₃-His-aac3. All cloned vectors were isolated by miniprep (Qiagen) and confirmed by PCR, restriction analysis, and sequencing (Cambridge Bioscience).

*Saccharomyces cerevisiae* strain WB-12 (MATa ade2-1 trp1-1 uro3-1 can1-100 aac1::LEU2 aac2::HIS3), lacking functional AAC1 and AAC2 carriers, was a gift from Dr. H. Terada (6). The AAC3 expression vector pYES-Pac2::NH₃-His-aac3 was transformed into the strain WB-12 using standard techniques, and transformants were selected on SC medium minus Trp plates (Invitrogen).

*Cell Culture and Preparation of Mitochondria—*A preculture of 500 ml was used to inoculate 30 liters of YM medium containing 3% glycerol and 2% ethanol in a 40 liter Bioreactor (FT Applikon). The cells grown at 30 °C were harvested at A₅₅₀ of 0.16 by centrifugation at 4,000 × g for 20 min. The cell pellet was resuspended in 0.85 M sorbitol, 0.1 M Tris, pH 8.0, 0.005 M EDTA, 0.2% bovine serum albumin at a volume ratio of 1:1. Phenylmethanesulphonyl fluoride was added at a final concentration of 1 mM immediately prior to cell disruption at 30,000 p.s.i. in a Z-Plus 2.2-kilowatt cell disruptor (Constant Systems). The suspension was centrifuged at 3,000 × g for 20 min at 4 °C (Sorvall) to remove cellular debris and unbroken cells, and the mitochondrial fraction was collected by ultracentrifugation at 140,000 × g for 4 °C for 1 h. The mitochondrial pellet was washed in 0.65 M sorbitol, 0.02 M Tris, pH 7.4, collected by ultracentrifugation at 140,000 × g for 4 °C for 1 h, and resuspended in the same buffer at a final concentration of 35 mg/ml.

*Liganding and Purification of Yeast AAC3—*The mitochondrial fraction (500 mg of protein) was incubated for 20 min at 4 °C with atractyloside (ATR) (1) (Calbiochem) at a concentration of 20 nmol of ATR per mg of protein (99% occupancy). Solubilization was carried out in 10 mM

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Extensive washing in buffers, consisting of 10 mM Tris, pH 7.4, 150 mM acetic acid Superflow (Qiagen) at a flow rate of 0.5 ml/min, followed by dialysis of a micellar mixture containing 1.5 mg/ml protein and 1,2-dioleoyl-sn-glycerol-3-phosphocholine solubilized in DDM at lipid to protein ratios of 0.2–0.45 w/w. The detergent removal of 100-μl samples was performed in Slide-a-Lyzers (Pierce) against 25 mM MES, pH 6.0, 100 mM NaCl, 10 mM EDTA, 2 g Biobeads, 5 mM NaN₃. Crystals were obtained after 7 days at 8°C.

Two-dimensional Crystallization—Crystallization was performed by dialysis of a micellar mixture containing 1.5 mg/ml protein and 1,2-dioleoyl-sn-glycerol-3-phosphocholine solubilized in DDM at lipid to protein ratios of 0.2–0.45 w/w. The detergent removal of 100-μl samples was performed in Slide-a-Lyzers (Pierce) against 25 mM MES, pH 6.0, 100 mM NaCl, 10 mM EDTA, 2 g Biobeads, 5 mM NaN₃. Crystals were obtained after 7 days at 8°C.

Electron Microscopy and Image Processing—Electron microscopy was performed on a 200 keV Tecnai F20 microscope with field emission gun and Gatan 626 cryo-stage cooled by liquid nitrogen. Images were collected at a magnification of 62,000 under standard low dose conditions. Images containing crystalline areas were selected by optical inspection, 2% of total, was a degradation product of the carrier as confirmed by Western blot analysis. The total yield of purified AAC3 was ~150 μg/liter of yeast culture.

Detergent removal from micelles containing purified ATR-inhibited AAC3, dioleoylphosphatidylcholine and dodecyl maltoside resulted in the formation of two-dimensional crystals in the form of tubes (Fig. 1). The tubes were variable in length, but always had a defined width of 0.25 μm (Fig. 1). The majority of these tubes seemed to grow from large vesicles, which most flattened tubes had two crystalline layers, but the average crystalline area was relatively small, i.e. 0.2 by 0.3 μm. For collection of high resolution data, images of frozen hydrated crystals were recorded by electron microscopy at liquid nitrogen temperatures. Fig. 2 shows the calculated image diffraction pattern of one crystalline area of good quality. Good spots extended to 8 Å with weaker more sporadic reflections to 5-Å resolution. The systematic absence of (h, 0) and (0, k) reflections, when h and k were odd numbers, indicated the existence of 2-fold screw axes in both directions (Fig. 2).

Images were processed and a projection map was calculated to 8-Å resolution (Fig. 3 and Table I). The total molecular footprint area covered by the protein density is 1,006 Å². The average projected area per helix in archeal rhodopsins, rhodopsin, and bacterial transporters calculated in the same way is 129 Å² (20–25). Therefore, the protein density could contain around 8 α-helices, but not many more because of helix-helix packing constraints (26). The projected structure thus repre-
sent a single AAC3 molecule. The unit cell contains four AAC3 molecules, and their density features are consistent with tilted α-helices seen in projection. The relative position and orientation of the molecules in the crystal are described by p222₁ symmetry and hence half of the molecules are oriented up and half down.

**DISCUSSION**

In this paper we present the projection structure at 8-Å resolution of the yeast ADP/ATP carrier embedded in the lipid bilayer. The projection structure is very different from those of bacterial transporters for which comparable structural information is available (22–24, 27–29). The AAC3 molecule consists of a continuous density in the form of a rounded triangle with two, possibly three, additional density peaks in the center (Fig. 4A). The density peaks on the ring are related to each other with pseudo 3-fold symmetry, which could be a reflection of the characteristic tripartite structure of mitochondrial carriers (4). The most consistent interpretation is that main structural scaffold of AAC3 consists of a bundle of six tilted transmembrane α-helices. The density peaks on the bundle most likely derive from tilted α-helices that partially overlap on the vertical as in bacteriorhodopsin (20). The proposed model agrees well with the total surface area of the molecule and would imply that both the amino and carboxyl terminus would be on the same side of the inner mitochondrial membrane (5–7). The projected length for each tilted α-helix in the bundle would be 13 Å, which compares well with an average value of 12.5 Å per tilted helix in bacteriorhodopsin (20). Alternative α-helical arrangements are extremely unlikely, because they are inconsistent with the pseudo 3-fold symmetry and tripartite repeats, the localization of both termini, average α-helical distances, and the surface area of the projected density.

The cross-sectional area in the center of the α-helical bundle is \(\sim 280 \, \text{Å}^2\), if one takes the side chains of the helices into account. Two, maybe three, additional density peaks are visible in the center of the bundle (Fig. 4A). The interpretation of these densities is less straightforward, but it is unlikely that the densities represent atractyloside or adenine nucleotides, because the density of these molecules is similar to that of amino acid side chains, which do not show in the density cut-off. Experiments with proteolytic enzymes, cross-linking, and labeling agents have shown that regions in matrix loops 1 and 2 are important in substrate recognition and in conformational changes induced by atractyloside binding (11, 16, 30). These regions have a propensity to form short amphipathic α-helices as can be inferred from secondary structure predictions (31).

### Table I

| Electron crystallographic data | \( p_{22,2} \) |
|-------------------------------|----------------|
| Plane group \(^a\)            | \( p_{22,2} \) |
| Unit cell dimensions \(^b\)   | \( a = 42.5 \pm 0.6 \, \text{Å} \) |
|                               | \( b = 172.1 \pm 2.3 \, \text{Å} \) |
|                               | \( \gamma = 90^\circ \) |
| Number of crystalline areas \(^c\)   | 5 |
| Range of defocus (Å)          | 200–12.0 |
| Number of unique reflections  | 552 |
| Overall phase residual \(^d\) | 460 ± 153 |
| Number of crystalline areas \(^e\)          | 12.1–9.5 |
| Resolution range (Å)          | 16.1° (43) |
| (number of reflections)       | 8.2 |
| Resolution range (Å)          | 28.4° (25) |
| (number of reflections)       | 9.6–8.2 |
| Resolution range (Å)          | 31.5° (19) |

\(^a\) Determined by ALLSPACE (33). For instance, for crystal 7501 the phase residual of symmetry-related phases was 10.4° compared to a target residual of 6.6°.

\(^b\) Determined values of the unit cell dimensions and S.D. for the five merged images. The values for all 40 processed crystals fell within this range.

\(^c\) Number of crystalline areas used for the merging. In total 90 images of untilted crystals were recorded and 40 were processed by the MRC image processing package (19). Images were selected for merging on the basis of the following criteria: the deviation of the dimensions from the average unit cell, the deviation of the included angle from 90°, the phase residual for pseudo \( p_{22,2} \), symmetry related phases, and the phase residual for phase comparisons of related reflections in image diffraction patterns of selected crystals.

\(^d\) Averaged phase deviations from 0 and 180° for the vectorally averaged phases.

\(^e\) Averaged in-plane temperature factor applied to restore high resolution amplitude fall-off scaled to bacteriorhodopsin.

\(^f\) Averaged phase deviations from 0 and 180° for the vectorially averaged phases in the indicated resolution range (45° random, IQ \(\leq\) 7).
These putative α-helices would basically be extensions of the even-numbered transmembrane α-helices on the matrix side linked with a short loop. It is not likely that these short α-helices enter the transmembrane region of the AAC3 molecule, because of α-helical distance constraints (26). The signature motif that is typical for mitochondrial carriers also has a propensity to adopt an amphipathic α-helical structure (31) and could be part of the odd-numbered transmembrane α-helices.

Many biochemical and biophysical studies support the idea that mitochondrial carriers are functional as homodimers. These studies include ultracentrifugation (8), cross-linking experiments (9–11), blue native gel electrophoresis (12), inhibitor studies (16), and differential tagging (13). The p22\textsubscript{2}:p22\textsubscript{2} crystal consists of symmetrical dimers organized in rows that are alternating in orientation (Fig. 3). A p22\textsubscript{2}:p22\textsubscript{2} crystal form was obtained under very similar crystallization conditions using ATR-inhibited AAC3 purified in Triton X-100 (data not shown). The structure was solved to 15-Å resolution from images of negatively stained two-dimensional crystals. The p22\textsubscript{2}:p22\textsubscript{2} crystal form also consisted of rows of symmetrical dimers like the p22\textsubscript{2}:p22\textsubscript{2} crystal form, but the rows always had the same orientation. The symmetrical dimer thus seems to be the crystallizing unit in both crystal forms, but this could simply be the preferred way in which AAC3 molecules crystallize. However, the dimer could also be the representation of the oligomeric state of the carrier in the membrane as observed in the aforementioned biochemical and biophysical studies. We cannot discriminate between these two possibilities, but the projection structure does prove that AAC3 molecules do not form an integrated 12 α-helical bundle as has been proposed (12). Nor do two molecules form at the interface a channel, through which a charged molecule like ATP or ADP could pass (14). The projection structure shows that nucleotides are most likely to pass through the membrane in the core of the monomer. If ADP/ATP transport requires cooperative interaction of two carriers (14), then this must occur by association of two separate monomers. Nucleotide exchange would in that case be a process in which two monomers each bind an adenine nucleotide, associate, change conformational state, release substrates, and dissociate for another round of transport. However, the central cavity in the molecule could in principle be large enough to bind two adenine nucleotides depending on the orientation of the substrates (Fig. 4, A and B). This opens the possibility that ADP/ATP exchange is carried out by a single carrier molecule.

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FIG. 4. Projection density map of a single ATR-inhibited AAC3 molecule. A, nine density peaks, positioned with pseudo 3-fold symmetry, are found on the continuous density ring with two, possibly three additional densities in the center of the bundle, B, for size comparison. Four different orientations of a single ATP molecule are shown in space-filling representation. The bar corresponds to 10 Å.