The ATP-binding cassette half-transporter Mdl1 from *Saccharomyces cerevisiae* has been proposed to be involved in the quality control of misassembled respiratory chain complexes by exporting degradation products generated by the m-AAA proteases from the matrix. Direct functional or structural data of the transport complex are, however, not known so far. After screening expression in various hosts, Mdl1 was overexpressed 100-fold to 1% of total mitochondrial membrane protein in *S. cerevisiae*. Based on detergent screens, Mdl1 was solubilized and purified to homogeneity. Mdl1 showed a high binding affinity for MgATP (*K*ₐ = 0.26 μM) and an ATPase activity with a *K*ₘ of 0.86 mM (Hill coefficient of 0.98) and a turnover rate of 2.6 ATP/s. Mutagenesis of the conserved glutamate downstream of the Walker B motif (E599Q) or the conserved histidine of the H-loop (H631A) abolished ATP hydrolysis, whereas ATP binding was not affected. Mdl1 reconstituted into liposomes showed an ATPase activity similar to the solubilized complex. By single particle electron microscopy, a first three-dimensional structure of the mitochondrial ATP-binding cassette transporter was derived at 2.3-nm resolution, revealing a homodimeric complex in an open conformation.

The ATP-binding cassette (ABC) superfamily constitutes a large class of active transporters found in all kingdoms of life. They couple the hydrolysis of ATP to the transport of substrates across membranes. ABC transporters are composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The TMDs form the translocation pore, whereas the NBDs are responsible for ATP binding and ATP hydrolysis, thus energizing substrate transport (1, 2). In prokaryotes and archaea, they can function as importers together with a substrate-binding protein, but generally ABC transporters operate as exporters. The TMDs and NBDs are found as homo- or heterodimers and can be arranged in any combination, e.g. as separate polypeptides, as single polypeptides, or as half-size transporters with fused TMD and NBD. The NBDs contain the highly conserved Walker A and B motifs as well as the C-, H-, and D-loops. Structural and biochemical data demonstrated that ATP binding induces dimerization of two NBDs in a head-to-tail orientation leading to a structure where both ATPs are sandwiched on the interface of the dimer (reviewed in Ref. 3). Each of the two nucleotide-binding sites in the dimer is formed by the Walker A and B motifs from one NBD and C-loop residues of the other NBD.

The *MDL1* gene encodes a membrane protein of 695 amino acids with a TMD followed by a NBD (4). Hydrophobicity and sequence homology analysis suggested that the TMD contains six transmembrane helices with the N and C termini located in the matrix. The minimal functional unit has been suggested to be a homodimer residing in the inner mitochondrial membrane (5). Mdl1 is a component of the mitochondrial quality control system and is involved in translocation of peptides with a length of 6–20 amino acids from the matrix into the intermembrane space (5). These products are generated by degradation of non-assembled inner mitochondrial membrane complexes by m-AAA (matrix-oriented ATPases associated with a variety of cellular activities) proteases (6). Although Mdl1 is not essential for cell viability, it is tempting to speculate that it plays a physiological role in the communication between the mitochondrial and the cellular environment. Homologues of Mdl1 have been identified in mammals, e.g. ABCB8 (M-ABC1) and ABCB10 (ABC-me, M-ABC2) (7–9). Several other functions for Mdl1 have, however, been suggested, e.g. Mdl1 partially restores Atm1 function (10). The ABC transporter Atm1 serves as a link between mitochondrial and cytosolic iron homeostasis, presumably by exporting iron-sulfur clusters assembled in the mitochondrial matrix into the intermembrane space (11). Overexpression of Mdl1 in Δatm1 cells results in a reduction of mitochondrial iron content and decreased sensitivity to H₂O₂ and transition metal toxicity, thus suggesting a role for Mdl1 in the regulation of cellular resistance to oxidative stress.

Here, we report the overexpression, purification, and functional reconstitution into proteoliposomes of Mdl1, which
Fingerprint of the ABC Transporter Mdl1

allowed us to study the biochemical properties of a mitochondrial ABC transporter for the first time. In addition, the three-dimensional structure of the ABC transport complex was obtained at 2.3-nm resolution by electron microscopy and single-particle analysis.

EXPERIMENTAL PROCEDURES

Materials—8-Azido-[α-32P]ATP was purchased from MP Biomedicals. Peptide libraries were synthesized by Fmoc (N-(9-fluorenylmethoxycarbonyl) solid phase chemistry (12). For immune detection, rabbit antisera were raised against the C terminus of Mdl1 (KGGVIDLDN5VAREV) and affinity-purified. Protein concentrations were determined using the Bradford assay (Pierce). Digitonin was purchased at Calbiochem, and all other detergents were from Anatrace.

Cloning—By the use of unique restriction sites, Mdl1 was systematically divided in three cassettes, facilitating exchange between different constructs. The cassettes were amplified by PCR from genomic DNA. Cassette I encodes the N terminus of Mdl1 until a silent Clal site, which was introduced at the nucleotides corresponding to residue S221. Cassette II includes the central region of Mdl1 from the Clal site to an intrinsic and unique BamHI site comprising amino acids Ser221–Lys422. Cassette III encodes the C-terminal including an additional His8 tag. A variant of cassette III was cloned without a C-terminal tag and named IIIa. Cassette I was also constructed without the first 68 amino acids, predicted to include the mitochondrial leader sequence, and named Ia. Primers used to generate the cassettes are described in the on-line supplemental material (supplemental Table S1). The cassettes I, II, and III were sequentially ligated into PET401 (13) resulting in vector pMDL1(pre).

Construction of Expression Plasmids—The MDL1 gene from vector pMDL1(pre) was cloned in different cloning and expression vectors for either Escherichia coli or Lactococcus lactis (supplemental Table S2). To integrate the MDL1 gene behind the strong PDR5 promoter, a PCR fragment was created by using pMDL1(pre) as template together with the pc4(f) and pc4(r) primers. The resulting PCR fragment was digested with Ascl and Pacl and ligated into pA6a-His3MX6 (14). This vector was used as a template to generate a PCR fragment with the primers pc5(f) and pc5(r), which was then used for homologous recombination in the Saccharomyces cerevisiae strain YALF-A1 (15). To generate the yeast overexpression vector, the MDL1 gene from vector pMDL1(pre) was amplified by PCR using the primers pc6(f) and pc3(r) and cloned into pYES2.1/V5-His-TOPO-Mdl1fl. pYES2.1/V5-His-TOPO-Mdl1fl_EQ, or pYES2.1/V5-His-TOPO-Mdl1fl_HA and grown overnight in SC medium (24), supplemented with amino acids without uracil and 2% glucose. The cells were harvested and resuspended to an A600 of 0.4 in SC medium supplemented with amino acids without uracil and 2% galactose. Growth was continued at 30 °C, and cells were harvested by centrifugation 8 h after induction. The mitochondria were isolated as described (25). The proteins were separated on SDS-PAGE (10%), and the expression levels were evaluated by Coomassie staining and quantitative immunoblotting.

Detergent Screens—To test the solubilization efficiencies, the mitochondria (5 mg/ml) were solubilized for 30 min in 100 μl of buffer S (50 mM Tris–HCl, 150 mM NaCl, 15% (v/v) glycerol, 2 mM imidazole, pH 7.5) supplemented with DDM, FC-14, or digitonin at ρ values of either 15 or 50 (Equation 1; where [lipid] is 0.1 times (w/w) the protein concentration, the average molecular mass of a lipid was taken as 875 Da, and CMC is the critical micelle concentration).

\[
\rho = \frac{[\text{detergent}]}{[\text{lipid}]} - \text{CMC}
\]

After ultracentrifugation (100,000 × g, 30 min, 4 °C), solubilized and nonsolubilized Mdl1 were compared by immunoblotting using an Mdl1-specific antibody. Chemiluminescent signals were quantified, and the percentage of solubilized protein was calculated. To analyze the stability and oligomeric state of Mdl1 mitochondria (0.5 mg of protein in 100 μl of final volume) were solubilized in different detergents (1%) each. Solubilized proteins were separated by ultracentrifugation (100,000 × g, 30 min, 4 °C) and analyzed by Blue native electrophoresis (gradient 6.0 to 16.5%) as described (26). Thyroglobulin (440 kDa), catalase (232 kDa), and lactate dehydrogenase (140 kDa) were used as markers.

Purification of Mdl1—Mitochondria (5 mg/ml) were solubilized in buffer S supplemented with 1% digitonin (ρ value of 15) and EDTA-free complete protease inhibitor mixture (Roche Applied Science) for 30 min at 4 °C under gentle shaking.
uble and insoluble fractions were separated by ultracentrifuga-
tion (100,000 g, 30 min, 4 °C), and the supernatant was
applied to an IDA-column (GE Healthcare) loaded with Ni2+
equilibrated with IMAC buffer (50 mM Tris-HCl, 150 mM
NaCl, 15% (v/v) glycerol, 2 mM imidazole, 0.1% digitonin, pH 7.5). The column was washed with 60 and 120 mM imidazole.
Specifically bound protein was eluted in IMAC buffer contain-
ing 200 mM imidazole. The protein was identified by immuno-
blotting and matrix-assisted laser desorption/ionization time-
of-flight peptide mass fingerprint analysis after trypsin
digestion, using Mascot (data base NCBInr 20040521).

**Nucleotide Binding Assays**—Nucleotide binding was measured
by 8-azido-[α-32P]ATP photo cross-linking experiments. Purified Mdh1 (0.5 μM) was incubated with increasing concen-
trations of 8-azido-[α-32P]ATP for 5 min on ice and UV-irradi-
ated for 5 min. For concentrations of 10 μM and above, 8-azido-
[α-32P]ATP was supplemented with nonradioactive
8-azido-ATP. Subsequently, samples were analyzed by SDS-
PAGE (10%). The gels were dried and exposed to a Kodak
8-azido-ATP Hydrolysis Assay—The ATPase activity was determined
equally frozen in liquid ethane. The samples were prepared in a
freeze fracture unit (400T Balzers) and shadowed with plati-
num/carbon at an angle of 45°. Replicas reinforced by pure car-
oblenegative with 4.8 mM
8-azido-ATP (Eq. 3).

\[
K_d(\text{ATP}) = \frac{\text{IC}_{50}}{1 + \left(\frac{\text{IC}_{50}}{\text{IC}_{50}}\right)}
\]

Applying the Cheng-Prusoff equation (27), the dissociation
constant \(K_d\) for ATP was derived from the IC_{50} value for ATP and the \(K_d\) value for 8-azido-ATP (Equation 3).

\[
K_d(\text{ATP}) = \frac{\text{IC}_{50}}{1 + \left(\frac{\text{IC}_{50}}{\text{IC}_{50}}\right)}
\]

**Peptide Transport Assays**—Iodination of peptide libraries and
peptide transport was performed as described (30). Proteo-
liposomes (0.5 μg of Mdh1) were preincubated with 4.8 mM
ATP and 10 mM MgCl2 in 50 μl of transport buffer (50 mM Tris, 25 mM KCl, 0.1 mM Dithiothreitol, pH 7.5) for 1 min at 30 °C.
The transport reaction was started by the addition of 125I-la-
beled X8 or X23 peptide libraries to a final concentration of 2
μM. The reaction was stopped after 6 min at 30 °C by the addi-
tion of 500 μl of ice-cold transport buffer supplemented with 10
mM EDTA. After centrifugation, the pellets were washed, and
radioactivity was quantified by γ-counting. To determine back-
ground activities, the samples were treated with apyrase (1 unit)
instead of MgATP.

**Single Particle Electron Microscopy**—Purified Mdh1(H631A)
was concentrated (Amicon Ultra-4; molecular weight cut-off
100 kDa) to 1 mg/ml, subjected to gel filtration (Superdex 200
PC 3.2), and equilibrated with buffer G (50 mM Tris-HCl, 150
MgCl2, 0.1% digitonin, pH 7.5). Peak fractions containing

7.5) at a concentration of 4 mg/ml. The lipid suspension was
extruded (Avanti Mini-Extruder) 11 times through a 0.4-μm
polycarbonate filter (Avestin). The liposome suspension was
extruded to a final DDM concentration of 4.2 mM, mixed with
solubilized Mdh1 in a molar lipid-to-protein ratio of 200, and
incubated for 30 min at 20 °C. Polystyrene beads (Bio-beads
SM-2; Bio-Rad) were washed three times with methanol,
washed five times with buffer R, and incubated with E. coli total
lipids (4 mg/ml in buffer R) before use. These Bio-beads (80
mg/ml final) were then added to the protein/detergent/lipid
mixture and incubated overnight at 4 °C. Bio-beads were
replaced by freshly prepared beads (80 mg/ml) and incubated
for 1 h at 4 °C. After removal of the Bio-beads, proteoliposomes
were pelleted by ultracentrifugation (190,000 × g, 1 h, 4 °C),
resuspended in buffer R, and applied to a sucrose step gradient (10,
20, and 30% (w/v)), and centrifuged (100,000 × g, 18 h, 4 °C).
The sucrose concentration of each fraction was determined with
an Abbé refractometer. The lipid concentration was deter-
mined as described (29). Briefly, 100-μl samples were mixed
with the same volume of 70% (v/v) perchloric acid, and after
incubation for two hours at 200 °C, 700 μl of reagent C (2.5% (w/v)
ammonium molybdate, 12.5% perchloric acid) and 700 μl of 3% (w/v) ascorbic acid were added. Finally, the samples were
incubated for 5 min at 100 °C, and absorbance at 820 nm
was measured and compared with a phosphate standard (Sigma-
Aldrich). The protein concentration in the different fractions
was determined by quantitative immunoblotting using isolated
Mdh1 as a reference. To elucidate the orientation of reconsti-
tuted Mdh1, proteoliposomes were treated with the protease
factor Xa at 10 μg/ml for 2 h at 25 °C in the presence of 1 mM
CaCl2.

**Freeze Fracture Electron Microscopy**—Proteoliposome prepa-
rations were placed between two small copper blades and rap-
idly frozen in liquid ethane. The samples were prepared in a
freeze fracture unit (400T Balzers) and shadowed with plati-
num/carbon at an angle of 45°. Replicas reinforced by pure car-
oblenegative with 4.8 mM

8-azido-ATP (final concentration, 0.5 μM) and incubated for 10 min at 30 °C. The reaction was stopped by the addition of 1 ml of reagent A (10 mM ammonium molybdate in 1 N HCl), 15 μl of 20 mM
H3PO4, and 2 ml of reagent B (isobutanol, cyclohexane, ace-
tone, and reagent A in a ratio of 5:5:1:0.1). The mixture was
vortexed vigorously for 30 s. After phase separation, 1 ml of the
organic phase was mixed with scintillation fluid (Microscint;
Packard BioScience), and the release of inorganic phosphate
was determined by β-counting (Beckman LS 6500 liquid scin-
tillation counter). \(V_{\text{max}}\) and \(K_m\) were derived by fitting the data
to the Michaelis-Menten equation. A similar protocol was used
to determine the ATPase activity of reconstituted Mdh1.

**Reconstitution of Mdh1—E. coli** total lipids (Avanti Polar Lip-
ids) were resuspended in buffer R (50 mM Tris, 25 mM KCl, pH

3953
Md11 were pooled, applied on a carbon grid, and stained with 2% uranyl acetate.

Electron microscopy (EM) images were recorded on film under low dose conditions in a FEI F30 electron microscope.

### TABLE 1
**Expression level of Md11 in various hosts**

|                      | S. cerevisiae wild type | E. coli | L. lactis | S. cerevisiae*
|----------------------|-------------------------|---------|-----------|------------------|
| Amount/total membrane protein (w/w) | 0.01                   | 0.005   | 0.01      | 1                |

*Mitochondrial membranes.

For three-dimensional reconstruction, tilt pairs were collected at 0° and 48° tilt and corresponding micrographs digitized on a Zeiss SCAI Scanner with 7-μm pixel size. Particle images were analyzed using the SPIDER package (31). XMIPP software (32) was used for classification of untitled images based on the Kohonen neural network. The maps were obtained from a total data set of $2 \times 1404$ projections by the random conical tilt method (33). The 2-fold symmetry typical for homodimers was imposed after verification at the latest step. After multiple refinements of all three Euler angles, the final volume was computed. The resolution was determined by the Fourier shell correlation criterion (cut-off at five times the noise correlation, FSC$_{5}$. The atomic structure of the open MsB (Protein Data Bank code 1JSQ (34)) was fitted into the electron density map of Md11 using Chimera software (35).

**RESULTS**

To collect the first biochemical and structural data of a mitochondrial ABC transporter, we set out to screen optimal conditions for expression, solubilization, purification, and reconstitution of Md11. In *S. cerevisiae*, Md11 is expressed at low levels (36) and is almost unaffected by various stimuli (environmental stress) as shown by microarray techniques (db.yeastgenome.org). Quantitative immunoblotting using the isolated NBD of Md11 as reference (22) demonstrated that Md11 is expressed to approximately 0.01% (w/w) of total mitochondrial protein (Table 1). We first tested expression of Md11 in *E. coli*. High and low copy number plasmids with different inducible promotors were studied under varying growth conditions and in different strains. The expression level of Md11 was determined by quantitative immunoblotting as described above. To promote Md11 expression, chaperones were induced by growth at 42 °C either in the presence of 2% ethanol or 1 M sorbitol. In addition, we tested co-expression with rare tRNAs or the SecY, SecE, SecG, and YidC components of the *E. coli* translocase. However, none of these factors improved the expression level of Md11 (0.005% of total membrane protein; Table 1). Because *L. lactis* has been demonstrated to be a suitable host for overexpression of mitochondrial proteins (37), Md11

**FIGURE 1. Expression, solubilization, stability, and oligomeric state of Md11.**

A. Mitochondria isolated at different time points after induction were analyzed by SDS-PAGE (10%) followed by Coomassie staining (upper panel) and immunoblotting (lower panel). 15 μg of protein were applied per lane. This shows that Md11 was overexpressed 8 h after induction (*). B. Mitochondria (0.5 mg of total protein) were resuspended in 100 μL of buffer S supplemented with different detergents as indicated (μ values of 15 or 50). Solubilized (S) and non-solubilized proteins (pellet, P) were separated by ultracentrifugation (100,000 × g) and analyzed by SDS-PAGE (10%). Md11 was detected by immunoblotting (left panel). The right panel depicts a quantification of the data. C. Mitochondria (0.5 mg in 100 μL) were solubilized in detergents (1% each) as indicated. The details are given under “Experimental Procedures.” Solubilized proteins were separated by ultracentrifugation (100,000 × g) and analyzed by Blue native electrophoresis and immunoblotting. Throglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and lactate dehydrogenase (140 kDa) were used as markers. C$_{12}$E$_{8}$, octaethylene glycol monododecyl ether; HEGA-10, n-decanoyl-N-hydroxyethylenglcamide; OG, n-octyl-α-D-glucopyranoside; TDAO, n-tetradecyl-N,N-dimethylamine-N-oxide; UDM, n-undecyl-β-D-maltoside; TX-100, Triton X-100.
was also expressed in this gram-positive bacteria. Expression of Mdl1 under control of a strong, nisin-inducible promotor led to an ~2-fold higher expression level (0.01% of total membrane protein) compared with E. coli, but the expression level was still very low (Table 1).

Because only low expression levels were obtained in either E. coli or L. lactis, we initiated expression screens in S. cerevisiae. Mdl1 was cloned behind a strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (p426GPD) or strong glycerol-dependent AAC2 (ADP/ATP carrier protein) promotor (pYES3P_A2AAC2). Surprisingly, both approaches resulted in lower than intrinsic levels of Mdl1 (Table 1). Alternatively, Mdl1 was cloned behind the PDR5 promotor, which is under control of the PDR network in the S. cerevisiae strain YALF-A1 (15). This system has been successful in the expression of membrane proteins in yeast (38–40). However, also in this case, only reduced levels of Mdl1 were obtained. We next examined expression from a multicopy plasmid under the inducible GAL1 promotor. Here, Mdl1 was overexpressed 100-fold, reaching 1% of total mitochondrial protein (Table 1). Mdl1 expression was strongly time-dependent, reaching its maximum 8 h after induction and then decreasing (Fig. 1A). This finding indicates that high Mdl1 levels are disfavored and result in increased instability or degradation of the protein. The degradation pathway is presently not known and is the focus of future studies. However, using this expression system, high levels of Mdl1 suitable for functional and structural studies were obtained.

We subsequently tested various detergents at different concentrations (ρ value) for optimal solubilization of Mdl1 from isolated mitochondria. n-Tetradecylphosphocholine (FC-14) showed by far the highest solubilization efficiency (>70% at ρ = 50) followed by n-tetradecyl-N,N-dimethylamine-N-oxide (66%), sodium cholate (56%), Triton X-100 (53%), and finally DDM (48%), octaethylene glycol monododecyl ether (46%), digitonin (44%), n-octyl-α-D-glucopyranoside (42%), and n-decanyl-N-hydroxy ethylenglucamide (35%) (Fig. 1B). We then investigated the stability and oligomeric state of Mdl1 in different detergents by Blue native electrophoresis (Fig. 1C). Mdl1 migrated as a defined band at 250 kDa in Triton X-100, octaethylene glycol monododecyl ether, digitonin, n-undecyl-β-D-maltoside, DDM, and n-octyl-α-D-glucopyranoside. In case of digitonin, Mdl1 was found in an almost monodisperse state corresponding to a homodimeric complex. Only a very broad smear was observed if Mdl1 was solubilized in FC-14 (not shown).

Mdl1 was isolated by metal affinity chromatography utilizing a C-terminal His$_8$-tag. Purification was analyzed by SDS-PAGE and immunoblotting (Fig. 2A). Like many integral membrane proteins, Mdl1 migrates at lower molecular mass than predicted (62 kDa instead of 71 kDa). Mdl1 was further identified by peptide mass fingerprinting. Three detergents were selected for further analysis: FC-14 as the most efficient detergent for solubilization, DDM known to preserve active ABC transporters and often used in crystallization of membrane proteins (41), and digitonin reported to hold fragile membrane protein complexes together (42). 1 mg of FC-14-solubilized Mdl1 (>95% purity) was isolated from 6 liters of yeast culture, 0.2 mg in case of DDM and 0.1 mg for digitonin. For each detergent, the ATPase activity of purified Mdl1 was determined, and remarkable differences were observed (Fig. 2B). Based on its high ATPase activity and almost monodisperse state, Mdl1 solubilized and purified in the presence of digitonin was selected for further functional and structural analysis.

To examine the nucleotide binding properties of Mdl1, 8-azido-[α-32P]ATP photo cross-linking experiments were performed. The apparent affinity constant for 8-azido-ATP was determined to be 7 μM according to a Langmuir-type (1:1) binding curve (Fig. 3A). Photo cross-linking of Mdl1 by 8-azido-[α-32P]ATP (5 μM) was inhibited by MgATP with an IC$_{50}$ value of 0.45 μM (Fig. 3B). Based on the apparent affinity of 8-azido-ATP, a dissociation constant for MgATP of 0.26 μM was estimated. ADP, CTP, UTP, and GTP show a similar affinity pat-
tern (inhibition from 85 to 50%; Fig. 3C) as observed for the isolated NBD (22). AMP-PNP bound with a reduced affinity, demonstrating previous observations that AMP-PNP does not perfectly mimic ATP. As expected, AMP did not compete for 8-azido-ATP binding. Taken together, these results show that full-length Mdl1 has a nucleotide specificity similar to that of the isolated NBD (22).

We next analyzed the ATPase activity of the digitonin-solubilized Mdl1. The ATP hydrolysis showed Michaelis-Menten kinetics ($V_{max}$ of 2.3 $\mu$mol/min $^{-1}$·mg $^{-1}$, and a turnover rate ($k_{cat}$) of 2.6 ATP per second and a $K_m$ of 0.86 mM). The data were fitted to the Hill equation $y = (ax^n)/(b^n + x^n)$, resulting in a best fit ($R^2 = 0.98$) with a Hill coefficient ($n$) of 0.98. The H631A (triangles) and E599Q mutant (circles) showed only background ATPase activity. B, the H631A mutant bound ATP as wild-type (WT) Mdl1 as shown by 8-azido-[α-32P]ATP photo cross-linking. The experiments were performed as described in Fig. 3.

The ATPase activity of wild-type and mutant Mdl1. A, the ATPase activity of Mdl1 (0.1 mM) was measured as a function of the ATP concentration at 30 °C. Digitonin-solubilized wild-type Mdl1 (squares) is active in ATP hydrolysis with a $K_m$ of 2.6 ATP per second and a $K_m$ of 0.86 mM. The data were fitted to the Hill equation $y = (ax^n)/(b^n + x^n)$, resulting in a best fit ($R^2 = 0.98$) with a Hill coefficient ($n$) of 0.98. The H631A (triangles) and E599Q mutant (circles) showed only background ATPase activity. B, the H631A mutant bound ATP as wild-type (WT) Mdl1 as shown by 8-azido-[α-32P]ATP photo cross-linking. The experiments were performed as described in Fig. 3.
orders of magnitude higher than observed for the isolated NBD (43). Furthermore, the turnover rate of full-length Mdl1 is constant over a large concentration range (0.01–1 μM) in contrast to the isolated NBD, which is nonlinearly dependent on the NBD concentration.

To analyze the mitochondrial ABC transporter in its membrane-embedded state, Mdl1 was reconstituted into liposomes. Purified Mdl1 was mixed with DDM-destabilized liposomes composed of E. coli lipids. Subsequently, the detergent was removed by absorption to Bio-beads. Protein reconstitution was analyzed by sucrose gradient centrifugation. Reconstitution of Mdl1 into the liposomes is evident from the co-migration of protein and lipids (Fig. 5A). Mdl1-containing liposomes were recovered at 16% sucrose, whereas empty liposomes migrated at 11% sucrose.

FIGURE 5. Reconstitution of Mdl1. A, empty or Mdl1-containing liposomes were applied to a sucrose gradient centrifugation. The fractions were analyzed with respect to the amount of Mdl1 and phospholipids. Reconstitution was evident from co-migration of the lipid and Mdl1 fraction (16% sucrose). A molar phospholipid-to-Mdl1 ratio of 160 was determined. Empty liposomes migrate at 11% sucrose. B, proteoliposomes were analyzed by freeze fracture electron microscopy. A statistical analysis revealed a homogenous particle size distribution of 10 ± 2 nm in diameter.

FIGURE 6. ATPase activity and orientation of reconstituted Mdl1. A, the ATPase activity of reconstituted Mdl1 was measured for 10 min at 30 °C in presence of 5 mM MgATP. After reconstitution, 85% of the activity of the digitonin-solubilized Mdl1 (wild type) was recovered. The H631A mutant is shown as control. B, the orientation of reconstituted Mdl1 was determined by protease protection assays utilizing the Xa cleavage site at the C terminus of Mdl1. Mdl1-containing liposomes were incubated with factor Xa (10 μg/ml) for 2 h at 25 °C in buffer containing 1 mM CaCl2. Cleavage of the C-terminal His6 tag and thus orientation of Mdl1 was followed by an anti-His antibody. 20 ± 5% of reconstituted Mdl1 remain uncleaved, demonstrating that 80% of the NBDs are in an outside orientation. Triton X-100 (2%) was used to permeabilize the proteoliposomes (almost 100% cleavage). The data are derived from three independent measurements. The anti-Mdl1 antibody was used as loading control. WT, wild type; TX-100, Triton X-100.
Fingerprint of the ABC Transporter Mdl1

A

B

C

D

E

Fingerprint of the ABC Transporter Mdl1

A

B

C

D

E
Fingerprint of the ABC Transporter Mdl1

Mitochondrial ABC transporters are difficult to study because of the two-membrane architecture of mitochondria, problems associated with analyzing an export process, and the high abundance of other ATPases and carriers/transporters. This asks for an in vitro system with pure and active protein. By systematic screens, we have established the overexpression of Mdl1 and present the first functional and structural analysis of a mitochondrial ABC transporter. Driven by an inducible promotor from a multicopy plasmid, a 100-fold overexpression of Mdl1 was achieved in S. cerevisiae. After screening optimal conditions for solubilization, the transporter was purified to homogeneity via metal affinity chromatography. The functional properties of solubilized as well as membrane-reconsti-

FIGURE 7. Single particle electron microscopy of Mdl1. A, upper panel, representative electron micrograph of negatively stained single Mdl1 complexes. Scale bar, 100 nm. Lower panel, different views of the final reconstruction (2-fold symmetrized). Rotational transitions are defined by arrows and corresponding angles. B, first row, representative class averages (>50% of the data set) obtained after multireference alignments showing the preferred orientation. The imaginary z direction is parallel to the paper plane. Second row, corresponding symmetrized class averages Scale bar, 100 Å. Third row, corresponding surface view of the reconstruction with increasing threshold levels from left to right. C, resolution of the final three-dimensional reconstruction at 23 Å. Fourier shell correlation curve (thick solid line) and the five sigma threshold (thin solid line) are shown. D, comparison of Mdl1 and the crystal structure of the V-shaped MsbA dimer from E. coli. The homodimer of MsbA (Protein Data Bank code 1JSQ, in red) was fitted into the three-dimensional electron density map of Mdl1 depicted as translucent isosurface in yellow. White horizontal lines indicate the dimension of a membrane. The z axis is parallel to the scale bar and perpendicular to the membrane plane. E, sequence alignment of MsbA from E. coli, V. cholera, and S. typhimurium. MsbA from S. aureus and Mdl1 from S. cerevisiae demonstrate their high similarity (51%; Fig. 7E). Interestingly, it was found that exclusively the open form of E. coli MsbA fitted exactly in terms of dimensions and space (Fig. 7D). The potential nucleotide-binding domains appear to be separated by more than 50 Å (Fig. 7D, bottom). We therefore conclude that our reconstruction reveals a homodimeric Mdl1 complex in an open conformation with the NBDs clearly separated.

DISCUSSION

Mitochondrial ABC transporters are difficult to study because of the two-membrane architecture of mitochondria, problems associated with analyzing an export process, and the high abundance of other ATPases and carriers/transporters. This asks for an in vitro system with pure and active protein. By systematic screens, we have established the overexpression of Mdl1 and present the first functional and structural analysis of a mitochondrial ABC transporter. Driven by an inducible promotor from a multicopy plasmid, a 100-fold overexpression of Mdl1 was achieved in S. cerevisiae. After screening optimal conditions for solubilization, the transporter was purified to homogeneity via metal affinity chromatography. The functional properties of solubilized as well as membrane-reconsti-

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cles with incorporated protein particles are formed (Fig. 5B). A statistic analysis revealed that the diameter of the particles is homogeneous and roughly 10 ± 2 nm. Analysis of the protein and lipid content of the proteoliposomes demonstrated that Mdl1 was reconstituted at a molar lipid-to-protein ratio of 160. We next investigated the ATPase activity of Mdl1 embedded in membranes. Remarkably, 85% of the ATPase activity of the solubilized protein was recovered (Fig. 6A). Taking advantage of the factor Xe cleavage site in front of the C-terminal His<sub>8</sub> tag, protease protection assays revealed that 80 ± 5% of Mdl1 are reconstituted in an “NBD-out” orientation (Fig. 6B). In the range of error, this result is consistent with the 85% ATPase activity of solubilized Mdl1, demonstrating that solubilized and reconstituted Mdl1 have a similar ATPase activity.

Mdl1 was proposed to be involved in the peptide export from the mitochondrial matrix (5). However, direct transport assays have not been performed so far. Combinatorial peptide libraries were instrumental to decipher the substrate specificity of ABC transporters (44–47). We therefore examined peptide libraries instrumental to decipher the substrate specificity of the mitochondrial matrix (5). However, direct transport assays were reconstituted Mdl1 have a similar ATPase activity.

A three-dimensional volume at a final resolution of 23 Å (FSC<sub>0.14</sub>; Fig. 7C) was calculated from corresponding tilted particles. 2-Fold symmetry in the direction of the z axis and perpendicular to the membrane plane was enforced at the latest step (Fig. 7, A, lower panel; B, third row; and D). The dimensions of the volume were determined to 125 × 110 × 95 Å<sup>3</sup>. Again, the four density lobes found in prominent views with the z direction in the paper plane are easily recognizable independent of the surface threshold level (Fig. 7B, third row). Moreover, a V-shaped outline of the electron density surface pointed to a homodimeric state of Mdl1.

To investigate these findings in more detail, several MsbA-like proteins with solved crystal structures were compared with the calculated electron density map of Mdl1 (34, 54–56). A sequence alignment of MsbA from E. coli, V. cholera, and Salmonella typhimurium, Sav1866 from Staphylococcus aureus, and Mdl1 from S. cerevisiae demonstrates their high similarity (51%; Fig. 7E). Interestingly, it was found that exclusively the open form of E. coli MsbA fitted exactly in terms of dimensions and space (Fig. 7D). The potential nucleotide-binding domains appear to be separated by more than 50 Å (Fig. 7D, bottom). We therefore conclude that our reconstruction reveals a homodimeric Mdl1 complex in an open conformation with the NBDs clearly separated.

FIGURE 7. Single particle electron microscopy of Mdl1. A, upper panel, representative electron micrograph of negatively stained single Mdl1 complexes. Scale bar, 100 nm. Lower panel, different views of the final reconstruction (2-fold symmetrized). Rotational transitions are defined by arrows and corresponding angles. B, first row, representative class averages (>50% of the data set) obtained after multireference alignments showing the preferred orientation. The imaginary z direction is parallel to the paper plane. Second row, corresponding symmetrized class averages Scale bar, 100 Å. Third row, corresponding surface view of the reconstruction with increasing threshold levels from left to right. C, resolution of the final three-dimensional reconstruction at 23 Å. Fourier shell correlation curve (thick solid line) and the five sigma threshold (thin solid line) are shown. D, comparison of Mdl1 and the crystal structure of the V-shaped MsbA-dimer from E. coli. The homodimer of MsbA (Protein Data Bank code 1JSQ, in red) was fitted into the three-dimensional electron density map of Mdl1 depicted as translucent isosurface in yellow. White horizontal lines indicate the dimension of a membrane. The z axis is parallel to the scale bar and perpendicular to the membrane plane. E, sequence alignment of MsbA from E. coli, V. cholera, and S. typhimurium (Protein Data Bank codes 1JSQ, open form (34); 1PF4, closed form (54); and 1Z2R, closed form (55)), Sav1866 from S. aureus (2HYD (56)), and Mdl1 (gi 6323217). The Walker A and B motifs, the Q-, C-, P-, D-, and H-loops are boxed and colored according to chemical properties of the amino acid residues. The overall color code results from the BLOSUM62 score.

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DISCUSSION

Mitochondrial ABC transporters are difficult to study because of the two-membrane architecture of mitochondria, problems associated with analyzing an export process, and the high abundance of other ATPases and carriers/transporters. This asks for an in vitro system with pure and active protein. By systematic screens, we have established the overexpression of Mdl1 and present the first functional and structural analysis of a mitochondrial ABC transporter. Driven by an inducible promotor from a multicopy plasmid, a 100-fold overexpression of Mdl1 was achieved in S. cerevisiae. After screening optimal conditions for solubilization, the transporter was purified to homogeneity via metal affinity chromatography. The functional properties of solubilized as well as membrane-reconsti-
Fingerprint of the ABC Transporter Mdl1

...tuted Mdl1 in respect to ATP binding and ATP hydrolysis were characterized in detail. Finally, a first three-dimensional map of the mitochondrial ABC transporter was obtained by single particle EM analysis.

The choice of the detergent used for the solubilization and purification of Mdl1 turned out to be critical. First, reasonable amounts of Mdl1 must be extracted from the membrane, which is best achieved by FC-14, and second, the oligomeric state and activity of the protein must be preserved. After purification in digitonin, Mdl1 was found to be mostly monodisperse by Blue native electrophoresis and showed the highest ATPase activity ($V_{max} = 2.3 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$). The observed activity is higher than reported for the majority of ABC transporters, e.g. MRP ($0.46 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$), MsbA ($0.04–0.15 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$), or BmrA ($1.2 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) (57–59). Only Atm1 showed a similar ATPase turnover number of 127 min$^{-1}$ ($V_{max} = 1.9 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) compared with 156 min$^{-1}$ in case of Mdl1 (60). The high ATPase activity indicates that solubilized Mdl1 is in an active state. This conclusion is further supported by the observation that solubilized and reconstituted Mdl1 have a similar ATPase activity.

Mdl1 binds MgATP with a remarkably high affinity ($K_d = 0.26 \mu\text{M}$) in comparison with the isolated NBD ($K_d = 2 \mu\text{M}$) (22). This value is also 2 orders of magnitude lower as compared with other ABC transporters (44, 61–63). Moreover, the apparent affinity for MgATP is 3,000 times higher than the Michaelis-Menten constant for ATP hydrolysis ($K_m(\text{ATP}) = 0.86 \text{mm}$). This implies that ATP binding and ATP hydrolysis are distinct steps. Remarkably, the activity of the isolated NBD is strongly dependent on the NBD concentration, whereas the ATP turnover of the full-length Mdl1 is concentration-independent (not shown). This demonstrates that in the full-length complex, the association rate of the NBDs (dimer formation) is not rate-limiting for ATP hydrolysis because of the close proximity and high local concentration within the transport complex.

Although Mdl1 is highly active in ATP hydrolysis, we did not observe a peptide-stimulated ATPase nor a peptide transport activity. We propose that this is either due to the fact that Mdl1 recognizes a very specific subset of peptides, which is underrepresented in the peptide libraries, or that the substrate of Mdl1 is more complex than originally anticipated, including modifications or associated cofactors. These findings challenge the initial observation that Mdl1 functions as peptide exporter in mitochondrial quality control (5). As was found to be case for Atm1, extensive screens will be required to resolve the issue of substrate specificity of Mdl1.

In this work, a first three-dimensional map of homodimeric Mdl1 complex has been determined by electron microscopy and single particle analysis. Single-particle analysis has successfully been applied to other ABC transporters, demonstrating the power of this method for particles even below 0.5 MDa. The Mdl1 preparation method uses digitonin to extract the protein from the membrane, metal affinity purification, and gel filtration. This procedure yields monodisperse Mdl1 particles suitable for structure determination.

Mdl1 appears to be in a homodimeric state because the dimensions of the particle of 125 × 110 × 95 Å$^3$ are too small for a dimer of a dimer and too large for a monomer. This conclusion is consistent with data derived from Blue native electrophoresis, gel filtration, and the diameter of membrane-embedded Mdl1 particles (freeze fracture EM). Remarkably, reconstructions obtained for different ABC transporters so far have been interpreted diversely in terms of their oligomerization state. The full size transporters PDR5 (63) and CFTR (61) showed particle dimensions that fit (homo) dimers, whereas for particles of the full size transporter P-glycoprotein a monomeric state was consistent (64). Dimensions of the half-size transport complex TAP1/TAP2 and YvcC implicate heterodimeric or homodimeric structures, respectively (49, 53).

The Mdl1 structure is clearly different from the very recently solved x-ray structure of the ABC transporter Sav1866 from S. aureus, which is found in an outward facing conformation with the two NBDs in close contact (56). Taken together, this analysis provides the first three-dimensional map of a mitochondrial ABC transporter. The outline and overall dimensions of the reconstruction as well as its 2-fold symmetry indicate that the Mdl1 complex is formed by a homodimer in the open conformation. Comparison with ABC transporters operating as exporters suggests major differences in the organization of the transmembrane domains within the transport cycle. However, x-ray and EM structures represent snapshots of different states, and the different overall organization of the TMDs and arrangement of transmembrane helices indicate very small energy barriers between these forms. This may allow for accommodating a broad spectrum of transported substrates. The high ATPase activity compared with other ABC transporters as well as the preserved homooligomeric state of Mdl1 suggest that the open EM structure represents an active conformation within the catalytic cycle and not an inactive, stable state. Furthermore, detergent artifacts disrupting the oligomeric complex and thereby forming new packing contacts of the TMDs can be excluded. Based on this structural information on Mdl1 at low resolution, it would be highly eligible to obtain a high resolution structure of an ABC exporter, such as Sav1866, in an open inward facing conformation. The overexpression and straightforward purification of Mdl1 provide the basis for future studies on the structure and function of Mdl1.

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