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Production of a Human Mitochondrial ABC Transporter in *E. coli*.

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Abstract

Membrane proteins play important roles in health and disease. Despite their importance, the study of membrane proteins has been significantly limited by the difficulties inherent to their successful expression, purification, and stabilization once they have been extracted from the cell membrane. In addition, expression of human membrane proteins commonly requires the use of expensive and/or time-consuming eukaryotic systems, hence their successful expression in bacteria will be obviously beneficial for experimental research. Furthermore, since lipids can have critical effects on the activity of membrane proteins and given the composition similarities between the inner mitochondrial membrane and the bacterial plasma membrane, production of mitochondrial membrane proteins in *E. coli* represents a logical choice. Here, we present a novel protocol to produce a human mitochondrial ATP-Binding Cassette (ABC) transporter in *E. coli*. The function of the three known human mitochondrial ABC transporters is not fully understood, but X-ray crystallography models of ABCB10 produced in insect cells are available. We have successfully expressed and purified ABCB10 from *E. coli*. The yield is close to that of another bacterial ABC transporter routinely produced in our laboratory under similar conditions. In addition, we can efficiently reconstitute detergent purified ABCB10 into lipid nanodiscs. Measurements of ATPase activity of ABCB10 produced in *E. coli* show an ATP hydrolysis rate similar to other human ABC transporters. This novel protocol facilitates the production of this human mitochondrial transporter for biochemical, structural, and functional analysis, and can likely be adjusted for production of other mitochondrial transporters.

Graphical Abstract

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Authors contribution:

Alexandra D. Saxberg: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing- Review & editing. **Melissa Martinez:** Validation, Formal analysis, Investigation. **Gregory A. Fendley:** Methodology, Validation, Investigation. **Maria E. Zoghbi:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing- Original draft, Writing- Review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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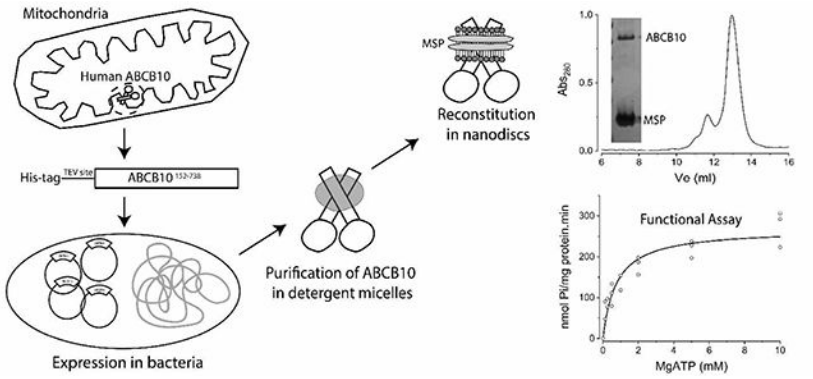

Keywords

ATP-binding cassette transporter; purification; mitochondria; human membrane protein; ATPase activity; nanodiscs

Introduction

ATP-binding cassette (ABC) transporters constitute the largest family of transmembrane proteins, which use energy from ATP to move a large variety of substrates across the plasma membrane and the membrane of intracellular organelles. These transporters are involved in many physiological functions, and mutations in their genes can cause diseases such as cystic fibrosis, Stargardt disease, X-linked sideroblastic anemia and defects in cholesterol and bile transport (1). ABC transporters are formed by two transmembrane α-helical domains that constitute the pathway for substrate translocation, and two highly conserved nucleotide-binding domains that bind and hydrolyze ATP. Humans have 48 ABC genes divided in eight subfamilies (A to G), according to their amino acid sequence similarities and protein architecture, and most of these proteins are transporters (2). Most human ABC transporters are expressed as full-length proteins, where the four domains are part of a single polypeptide, whereas seven members of the B family, four of the D family, and five of the G family are expressed as “half-transporters” that must dimerize to be functional (2, 3). X-ray crystallography and Cryo-electron microscopy studies have provided abundant structural information about ABC transporters, leading to a variety of proposed molecular mechanisms for their transport cycle (4–6). Given the great diversity of ABC transporters and the apparent lack of a one-size fits all mechanism, further investigations including as-yet unstudied ABC transporters are needed to achieve a better understanding of these important proteins.

The biochemical and structural analysis of transmembrane proteins commonly involve heterologous overexpression, extraction of the proteins from the membrane with the use of detergents, followed by standard purification procedures. Until now, human ABC transporters, either full-length (ABCA4, ABCB1, ABCB4, ABCB11, CFTR) or half-transporters (TAP, ABCB10, ABCD4, ABCG2, ABCG5/ABCG8), have been expressed and purified from yeast, insect, or mammalian cells (7–16). However, protein expression in eukaryotic systems is more complex, time consuming, and expensive as compared to

prokaryotic systems (17). Therefore, we decided to explore the possibility of expressing and purifying a human ABC transporter in bacteria. The half-transporter ABCG2 has been shown to express in bacteria with variable results: inside-out vesicles showed ATPase and transport activity when expressed in *L. lactis* (18), but no activity when expressed in *E. coli.* (19). However, in these studies the transporter had not been purified. Here, we have used the human half-transporter ABCB10, which forms a homodimer in the mitochondrial inner membrane (20, 21), as our model system. X-ray crystallography structures of ABCB10, expressed and purified from insect cells, and information for the basal ATPase activity for this transporter are available (14). The cellular function and physiological substrate of ABCB10 are still unknown, but this transporter has been implicated in heme biosynthesis and protection of cells against oxidative stress (22, 23). *Abcb10* knockout mice died *in utero*, highlighting the vital role of this transporter (24, 25). Our ability to successfully express and purify ABCB10 in a prokaryotic system will facilitate further biochemical and structural analysis of this protein and open the doors to an efficient widespread mutational analysis that could help elucidate the molecular mechanism underlying the function of this transporter. In addition, our results suggest that it can be worth exploring the use of this methodology to produce other human ABC transporters, especially those present in mitochondria.

2- Materials and Methods

Bacterial Plasmids:

ABCB10 gene without mitochondrial targeting sequence (protein includes amino acids 152 to 738), as previously reported for expression in insect cells (14), was synthesized (Fisher Scientific) with a C-terminal TEV protease recognition site (ENLYFQG) followed by a poly-histidine-tag. Additionally, two ABCB10 genes (native and codon-optimized), also without mitochondrial targeting sequence, were synthesized (GenScript) with an N-terminal poly-histidine-tag followed by a TEV protease recognition site. Synthesized genes were flanked by recognition sequences for the restriction enzymes Nco I and BamH I used for gene insertion in the multiple cloning sites region of the pET19b vector (Novagen). The synthetic DNA powder was dissolved with nuclease-free water to a concentration of 200 nmol/μl and stored at −20°C.

Protein Expression in Bacteria:

E. coli competent cells BL21-CodonPlus (DE3)-RIPL (Agilent), Rosetta 2 (DE) (Novagen) or Lemo21(DE3) (New England BioLabs) were transformed with plasmid containing the ABCB10 gene under appropriate antibiotic selection. Four 3.8 L flasks, each containing 1 L of media complemented with 0.5% glucose, were inoculated with 6 ml of an overnight culture grown at 37°C and 250 rpm. When Luria Broth (LB) media was used for protein expression, cells were grown at 37°C and 250 rpm until OD_{600} was 0.4. At this point, shaking speed was decreased to 125 rpm and temperature was lowered to 30°C. Protein expression was induced with 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) when OD_{600} was 0.6. When Terrific Broth (TB) was used for protein expression, the procedure was similar, with the exception that temperature and rpm were lowered when OD_{600} was 0.6 and cells where induced when OD_{600} was 1. Cells were collected between 3 and 4 hours

after induction by centrifugation (4,000 g; 15 minutes in Sorvall Legend XFR, TX1000 rotor with adapter for 0.5 L conical bottles). Cell pellets from the four-liter cultures were resuspended in ~40 ml of a low salt buffer containing 100 mM NaCl, 10% glycerol, 20 mM TrisHCl pH 8 plus freshly added 1 mM phenylmethylsulphonyl fluoride (PMSF) and frozen at −80°C until ready to use. Expression in LB usually resulted in ˜20 g of wet cell pellet once cell pellets from the four liters were combined.

Protein Purification:

Membrane fraction: Cell pellets were thawed on ice and bacterial cells were lysed either with EmulsiFlex (Avestin) or by sonication after 1-hour treatment with 0.5 mg/ml lysozyme (Fisher Scientific) at 4°C with rotation. Effectivity of the treatment with lysozyme was evident by the increased viscosity of the cell suspension. Cell sonication was performed with a microtip (Misonix ultrasonicator) on ice, with bursts of 30 sec with 30 sec pauses between bursts for a total of 12 min (6 minutes of sonication time). Once cells were lysed, the ionic strength of the homogenate was increased by addition of an equal volume of high salt buffer containing 2 M NaCl, 10% glycerol, 20 mM TrisHCl pH 8 and 1 mM PMSF and incubated at 4°C with rotation for 10 minutes. Debris and unbroken cells were removed by low speed centrifugation (6,000 g; 15 minutes) in a Sorvall Legend XFR (Thermo Scientific) using a F14–6×250 fixed angle rotor with adapters for 50 ml conical tubes. The supernatant was recovered and centrifuged in a Sorvall WX ultracentrifuge (Thermo Scientific) using a F50L-8×39 rotor at 32,000 rpm (~109,000 g) for 1 hour, at 4°C. The pellets, which contain the membrane fraction, were transferred using a spatula to a Dounce homogenizer on ice containing 20 ml of resuspension buffer: 300 mM NaCl, 10% glycerol, 20 mM TrisHCl pH 8, 20 mM imidazole, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5 mM tris(2-carboxyethyl)phosphine (TCEP). The homogenized membrane suspension can either be used immediately for the solubilization step or kept in the freezer at −80°C for later use. The total protein concentration in the membrane suspension measured by bicinchoninic acid assay (BCA) was usually 8–10 mg protein/ml.

Solubilization: The ~20 ml of membrane suspension was combined with an equal volume of resuspension buffer containing 2% dodecylmaltoside (DDM, Anatrace) and 0.2% cholesteryl hemi-succinate (CHS, Anatrace) and incubated with rotation for 1 hour at room temperature. The solubilized membranes were centrifuged in a Sorvall WX ultracentrifuge (Thermo Scientific) using a F50L-8×39 rotor at 32,000 rpm (~109,000 g) for 45 min, at 4°C. The supernatant contains the solubilized membrane proteins of interest. CHS was either freshly dissolved in DMSO or prepared as a DDM/CHS stock (10%/1%) by sonication (Misonix ultrasonicator) and kept at 4°C.

Affinity Chromatography: 0.5 ml of Ni-NTA resin (1 ml of slurry; Qiagen) was first washed with water and then equilibrated with wash buffer (300 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 8, 20 mM imidazole, 0.06% DDM, 0.02% CHS, 0.5 mM TCEP). The ~40 ml of solubilized membrane suspension was incubated for 2 hours with the pre-equilibrated Ni-NTA resin, at 4°C on a rotating platform. Then, the Ni-NTA resin was loaded onto a 1 ml glass column (BioRad) while Abs_{280} and buffer conductivity were monitored with a low-pressure chromatography system (Biologic-LP, BioRad). Wash buffer was passed through

the resin at 0.5 ml/min until Abs_{280} returned to baseline value (~20 column volume). Protein was eluted with elution buffer containing 300 mM imidazole, 300 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.5, 0.06% DDM, 0.02% CHS, 0.5 mM TCEP) and 1 ml fractions were collected manually. Aliquots of each fraction (15 μl) were analyzed by 10% SDS-PAGE and the best fractions were pooled for the His-tag removal step.

His-tag removal: Usually, only the fractions with higher concentration of ABCB10, according to SDS gel, were pooled for a total volume of 3 ml. A 100 μl aliquot of the pooled fractions was buffer exchanged to remove imidazole (using a 0.5 ml desalting Zeba column; Thermo Scientific) and protein concentration was measured in a nanodrop (NanoDrop 2000 spectrophotometer, Thermo Scientific) using a molecular weight of 65.9 kDa and calculated extinction coefficient of 40,400 $M^{-1}.cm^{-1}$ (ExPASy Bioinformatics Resource Portal). TEV protease (2.5 mg/ml) in 200 mM NaCl, 10% glycerol, 2 mM ethylenedinitrilotetraacetic acid (EDTA), 10 mM 1,4-dithiothreitol (DTT) was added to the solubilized protein. Usually 0.3 ml of the TEV stock were added to 3 ml of ABCB10 pooled fractions. The proteolysis reaction was placed on a pre-washed dialysis bag (6–8 kDa molecular weight cut off; Spectrum Laboratories) and incubated overnight, at 4°C, in one liter of dialysis buffer (100 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.5, 0.06% DDM, and 0.5 mM DTT). Next day, to remove the his-tagged TEV protease and uncut ABCB10 protein, the sample was recovered and incubated with 0.2 ml of pre-washed Ni-NTA resin for 2 hours, at 4°C on a rotating platform. Supernatant containing purified ABCB10 was aliquoted and kept at −80°C until ready to use.

ABCB10 Expression and Purification in Insect Cells: A baculovirus carrying the gene for ABCB10 (amino acids 152–738) containing a C-term TEV protease recognition site followed by a poly-histidine-tag was made following recommended procedures (Invitrogen) and was used as a control for some experiments. ABCB10 was expressed in insect Sf9 cells, which were collected 3-days post-infection by low speed centrifugation (500 g for 5 minutes in Sorvall Legend XFR, F14–6×250 fixed angle rotor with adapters for 50 ml conical tubes). Protein was purified following a previously published protocol (14). Protein expressed in insect cells was used as positive control for Western blots, and protein purified from insect cells was used as control in circular dichroism experiments.

Circular Dichroism: ABCB10 folding and thermal stability was evaluated by circular dichroism (CD). Protein purified from insect cells or bacteria was diluted to a final concentration of 2.5 μM with buffer (100 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.4, TCEP 0.1 mM, 0.06% DDM) up to a total volume of 200 μL. CD spectra were recorded in a 1 mM width quartz cuvette (Starna Cells) using a J-1500 CD spectrometer (J-1500, Jasco). Samples were equilibrated for five minutes at the desired temperature before each acquisition.

Reconstitution in Nanodiscs: ABCB10 in detergent, expressed and purified from bacteria, was mixed with *E. coli* polar or total lipids (Avanti Polar Lipids) and membrane scaffold protein MSP1D1 at molar ratio of 1 transporter to 6 MSP1D1 and 360 lipids. Lipids in chloroform were aliquoted and dried under nitrogen gas. Dried lipids were stored at

−20°C and then resuspended at 20 mg/ml in buffer containing either 100 mM sodium cholate or 3% DDM following three freeze (−80°C)/thaw cycles, with water bath sonication after each cycle (Ultrasonic bath M1800, Branson). When sodium cholate was used, the final concentration of sodium cholate in the whole reconstitution mixture was adjusted to 20 mM (26). MSP1D1 (plasmid obtained from Addgene) was expressed and purified as described before (26). Detergent absorbing bio-beads (Bio-beads SM2 adsorbent media, BioRad) were pre-washed with methanol, ethanol, and water according to manufacturer's recommendations and stored at 4°C until ready to use. Bio-beads were added in two steps, with a two-hour incubation time between additions, to reach a final concentration of 0.8 g/ml, and the sample was incubated overnight at 4°C on a rotating platform. Next morning, the reconstituted sample was recovered from the bio-beads and centrifuged at 10,000 g for 10 minutes at 4°C. An aliquot of the supernatant was injected in a 24 ml size-exclusion chromatography column (Enrich 650, BioRad) equilibrated with 100 mM KCl and 20 mM Tris-HCl pH 7.5. The column was connected to an NGC Quest 10 chromatography system (BioRad) and chromatography was performed at room temperature and a flow rate of 1 ml/min. When sodium cholate was used for reconstitution, the whole reconstituted sample had to be concentrated (Vivaspin 6, 100 kDa cutoff, Thermo Scientific) and run through size-exclusion column to remove aggregates (see Results and Discussion). Empty nanodiscs, used as control, were reconstituted in a similar way but adding dialysis buffer to the reconstitution mix instead of purified ABCB10. Concentration of ABCB10 in nanodiscs was estimated from Coomassie-stained gels using aliquots of known concentration of ABCB10 in detergent as reference.

ATPase activity: The activity of the transporter purified from bacteria was measured with an enzyme-coupled ATPase assay, where the rate of hydrolysis of ATP by the protein is coupled to NADH oxidation, which can be measured as a decrease in absorbance at 340 nm (27). The cocktail buffer for the assay contained 100 mM KCl, 5 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM NaATP, 10 mM $MgCl_2$, 3 mM phosphoenol pyruvate, 0.8 mM NADH (OD_{340} should be close to 2.8). The cocktail was aliquoted and stored at −20°C until ready to use. Right before the experiment, 0.5 mM TCEP plus the enzymes pyruvate kinase and lactate dehydrogenase were added to the cocktail. All reagents and enzymes were from Sigma-Aldrich. For determination of K_m, the concentration of ATP in the cocktail was varied as desired. When the activity of ABCB10 in detergent was evaluated, the cocktail was also supplemented with 0.06 % DDM. The assay was carried out on 96-well UV-transparent flat bottom plates (Corning) where 20–30 μl of protein sample (~30 μg of transporter) and 180–170 μl of cocktail were added per well, for a final volume of 200 μl. The decrease in NADH absorbance was measured at 340 nm in a microplate reader (SpectroFluor, Tecan or SPECTROstar Nano, BMG Labtech) during 3 to 4 hours at 37°C. ATPase hydrolysis rates were calculated from the slope of the linear decay, using an extinction coefficient (ε) = 6220 $M^{-1}.cm^{-1}$ and a pathlength (l) = 0.52 cm. The slope of the light induced NADH degradation during the assay was determined in wells were empty nanodiscs were added (blank). The slopes of blanks with empty nanodiscs or just buffer were the same. The slope of the linear region of the plots was determined by a straight-line fitting using the software Origin (Microcal) or MARS data analysis software (BMG Labtech).

Western-Blot analysis: Expression of ABCB10 in insect cells of bacteria was confirmed by Western blot using a primary antibody against a region within amino acids 487–726 (in the nucleotide binding domain) of human ABCB10 (Thermo Scientific). A WesternDot-625 secondary antibody (Invitrogen) was used for detection and images were acquired in an E-gel Imager (Life Technologies).

3- Results and Discussion

Expression of ABCB10 in bacteria:

Western blot analysis using an antibody against human ABCB10 (Figure 1a) showed a similar molecular weight band in membrane fractions of both insect cells and bacteria expressing this transporter, indicating that ABCB10 can be expressed in *E. coli*. We found no obvious differences when ABCB10 was expressed in BL21 or Rosetta-2 cells. Additional expression tests on Lemo21 cells showed similar results. For our initial studies, we used the non-codon optimized gene with C-terminal His-tag, which resulted in a low expression level detected by Western-blot analysis, but not detectable by Coomassie stained gels. The gene with N-terminal His-tag significantly increased the expression of ABCB10 to a level that could be detected by regular Coomassie staining for both, the codon-optimized and the non-codon optimized sequence (Figure 1b). Therefore, this higher expression level appears to be the result of the presence of the N-terminal His-tag rather than an effect of codon-optimization. This higher expression level did not result in a higher yield of purified ABCB10: the level of transporter that can be extracted during membrane solubilization was similar in both cases, likely because an excess of synthesized transporter ends up in inclusion bodies. Despite not offering a yield improvement, the use of the gene with N-terminal His-tag offers an advantage due the obvious convenience of easily confirming protein expression by just Coomassie staining.

We also tested the effect of two different growth media (Luria Broth and Terrific Broth), as well as various temperature conditions (15, 20, 30 or 37°C) and incubation times (ranging from overnight at low temperature to up to 3 hours at 37°C). The use of Terrific Broth instead of Luria Broth resulted in an increased cell pellet mass, but it did not translate into higher protein purification yield. Figure 1c shows an example of the expression level at lower temperature, with the overexpression band corresponding to ABCB10 already evident at only 3 hours after induction. Figure 1d shows the effect of different levels of IPTG on protein expression, where a clear band was already observed after induction with 0.25 mM IPTG and 0.5 mM IPTG seems as effective as higher concentrations of the inducer. Figure 1d also shows the levels of expression at different times after induction. The level of expression and protein yield was similar for the different tested conditions; therefore, the simplest expression procedure was adopted: inducing with 0.5 mM IPTG for 3 hours at 30°C in Luria Broth (see details in methodology section).

ABCB10 Purification:

His-tagged ABCB10 expressed in *E. coli* was purified using standard affinity chromatography procedures. The Ni-NTA eluate fractions contained ABCB10 plus some undesired contaminant bands (Figure 2a). Neither increases in ionic strength nor imidazole

concentration during incubation/wash steps, or elution with an imidazole linear gradient, improved the purity of the eluate. Contaminant bands can be observed in the eluate even after washing the column with buffer containing 1M NaCl (figure 2b) or with buffer containing 100 mM imidazole (Figure 2c). High concentration of imidazole resulted in some loss of ABCB10, whereas several contaminant bands were not removed. Conveniently, the contaminant bands can be efficiently removed during a second Ni-NTA purification once ABCB10 has been treated with TEV protease to cleave off the His-tag from the transporter (Figure 2c). Since His-tag removal is already a necessary step to avoid possible interference of this tag on the activity of the transporter, this second affinity purification step plays a dual role: 1) separate un-cleaved ABCB10 and His-tagged TEV from tag-free ABCB10, and 2) remove contaminant bands. The purity of the final protein fraction (~95%, as judge from gels), makes unnecessary the use of a second purification tag. The yield was ~0.5 mg of transporter per liter of cell culture, which was not very different from the yield obtained in our laboratory for bacterial ABC transporters, like MsbA (~0.8 mg of transporter per liter of cell culture). Therefore, the human transporter ABCB10 can be expressed in bacteria and purified using procedures commonly employed for the purification of other membrane proteins.

ABCB10 Folding and Activity:

We also studied the folding and activity of purified ABCB10 in detergent micelles. Circular dichroism spectra (Figure 3a) showed that ABCB10 purified from bacteria is folded and, as expected, it becomes unfolded as temperature is increased. The spectra of ABCB10 purified either from bacteria or from insect cells are essentially indistinguishable (Figure 3b). The purified transporter also displayed ATPase activity at physiological temperature (Figure 3c). However, the linearity of the ATP hydrolysis rate was lost over the duration of the enzyme-coupled assay, suggesting that under these experimental conditions (detergent micelles, 37°C) the transporter becomes unstable. This inactivation is not surprising, given the importance of lipids for the stability and function of ABC transporters (16, 28, 29). The purified transporter in detergent micelles must be frozen in aliquots or reconstituted to minimize protein loss of function. Together, these data indicate that the human ABC transporter purified from bacteria is folded and the nucleotide binding domains are capable of binding and hydrolyzing ATP.

Reconstitution into nanodiscs:

The activity of ABC transporters can differ greatly when the transporters are studied in detergent micelles or when incorporated in a lipid bilayer. Detergent micelles are not a good mimic of a membrane and can lead to artifacts. Therefore, we decided to reconstitute the detergent-solubilized ABCB10 into a small lipid bilayer system known as nanodiscs (26). Once reconstituted, the ABCB10 transporter is surrounded by a discoidal phospholipid lipid bilayer, where the outer hydrophobic tails of the lipids are shielded from the aqueous environment thanks to the membrane scaffold protein (MSP) that forms a belt around the disc. We used MSP1D1 as the membrane scaffold protein, which yields nanodisc of ~10 nm diameter (26). *E. coli* lipids were used for reconstitution because their composition is relatively similar to that of the inner mitochondrial membrane, the native environment for ABCB10.

It is common to use sodium cholate as detergent for solubilization during nanodisc reconstitution procedures. We consistently found that size-exclusion chromatography elution profiles of ABCB10 reconstituted using sodium cholate showed a significant peak of aggregates (Figure 4a). The ABCB10 protein contained in this aggregates fraction had no obvious activity on ATPase assays. The profile also showed a peak for nanodiscs that contain catalytically active ABCB10, followed by a peak of empty nanodiscs. When DDM (3%) was used to solubilize the lipids instead of sodium cholate, there was also some protein aggregation. However, the aggregates were large enough to be easily removed by standard centrifugation of the sample before it was loaded onto the size-exclusion column. The elution profiles of ABCB10 reconstituted with DDM consistently showed no aggregates (Figure 4b), as the aggregated protein appears in the pellet of the pre-loaded sample (**inset**, Figure 4b). In this profile, we see the peak for nanodiscs that contain catalytically active ABCB10, followed by a peak of empty nanodiscs. We therefore conclude that using DDM for the reconstitution of ABCB10 allows a more convenient removal of protein aggregates from the nanodisc sample.

ATPase activity:

The reconstituted transporter showed a linear ATP hydrolysis rate during the duration of the enzyme-coupled assays (Figure 5a), suggesting greater stability of the protein incorporated in the membrane compared to detergent (see also Figure 3b). In fact, the reconstituted transporter can be stored at 4°C for several days without obvious negative impact on catalytic activity. The measured ATPase activity of reconstituted ABCB10 (Figure 5b) was close to 270 nmol Pi/mg of transporter*min, with a K_m of 0.7 mM for MgATP. These values are similar to those reported for ABCB10 purified from insect cells (14). Such basal ATPase activity is a common feature of ABC transporters, but this ATP hydrolysis rate can be stimulated by the presence of substrate or activator drugs (30). In addition, the basal ATPase activity of the protein reconstituted either following the standard protocol that involves use of sodium cholate, or our protocol using DDM were not different (Figure 5c). Therefore, ATPase assays of the nanodisc-reconstituted ABCB10 sample, as we report here, represent a promising and convenient system for the screening of putative substrates and/or modulator drugs of this orphan transporter.

4- Conclusion

In summary, the data show that a functional human mitochondrial ABC transporter can be expressed and purified using *E. coli*, at a fraction of the time and cost of using eukaryotic expression systems. Production of the protein in bacteria represents a great advantage, especially when the creation of multiple mutations is being considered. Creating a mutant in *E. coli* is fast and inexpensive. On the contrary, production of a mutant protein in insect cells requires the more expensive and time-consuming creation and replication of a new baculovirus. In addition, our data show that the transporter produced in bacteria can also be effectively reconstituted for functional assays. This strategy will significantly facilitate biochemical, mutational, and structural studies of this human protein of yet unknown function. In addition, we think that a similar approach can also facilitate the production of other human ABC transporters localized in mitochondria or even other organelles.

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Highlights

- The human transporter ABCB10 can be expressed and solubilized from *E. coli* membranes.
- The purified transporter is folded and displays ATPase activity at physiological temperatures.
- Reconstitution into a lipid bilayer improves stability of the transporter.

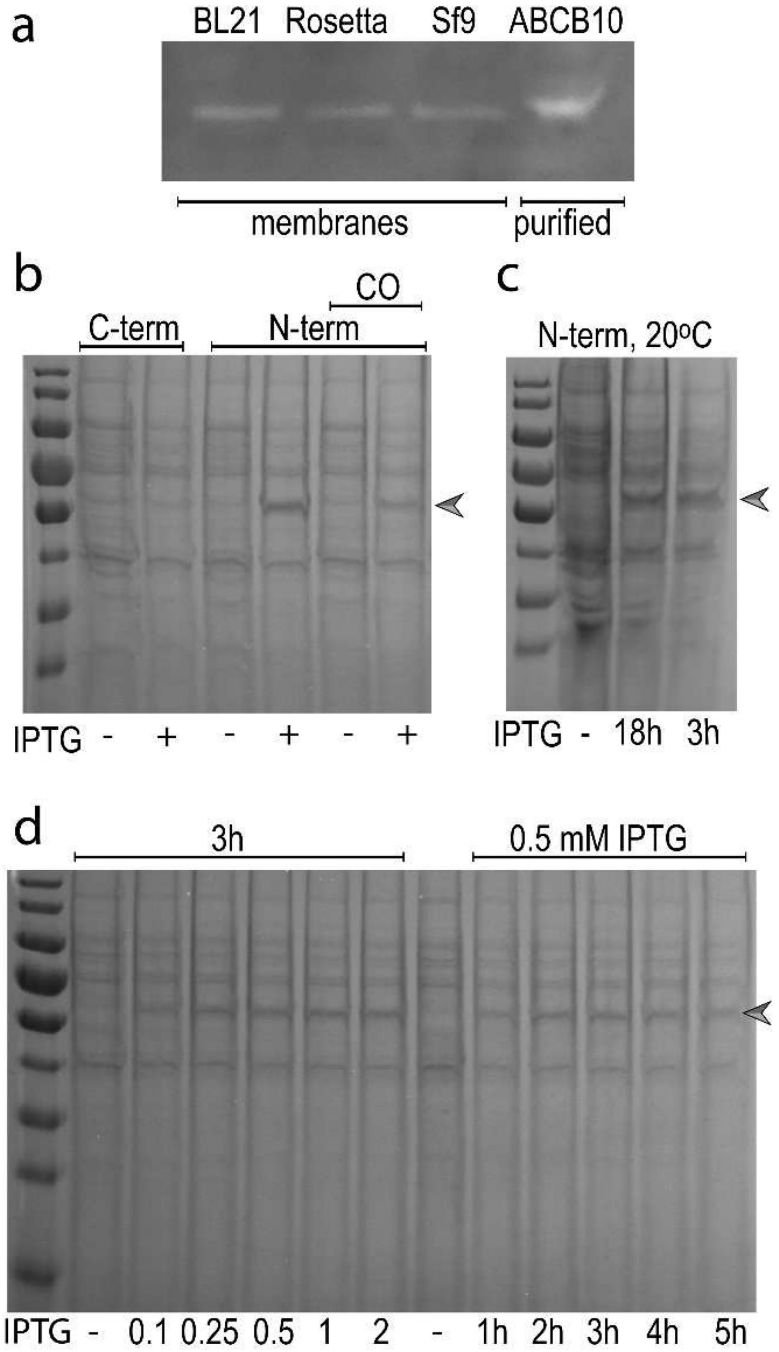

Figure 1.

Expression of ABCB10. a, Western blot of membrane fractions of bacterial cells (BL21 and Rossetta-2) or insect cells (Sf9) expressing ABCB10. The transporter purified from insect cells was used as a positive control. b, Coomassie-stained gel of bacterial cells non-induced (−) or after 3 hours induction with 0.5 mM IPTG (+) at 30°C. The His-tag was either in the C-terminus (C-term) or the N-terminus (N-term) of ABCB10. Expression of codon optimized (CO) ABCB10 with N-terminus His-tag is also shown. In this and other gels in this figure the arrowhead shows the position of a band corresponding to ABCB10. c,

Coomassie-stained gel of bacterial cells expressing N-terminus His-tagged ABCB10 at 20°C, 3 and 18 hours after induction with 0.5 mM IPTG. d, Coomassie-stained gel of bacterial cells expressing N-terminus His-tagged ABCB10 at 30°C, either 3 hours after induction with different IPTG concentrations (in mM) or at different times (in hours) after induction with 0.5 mM IPTG.

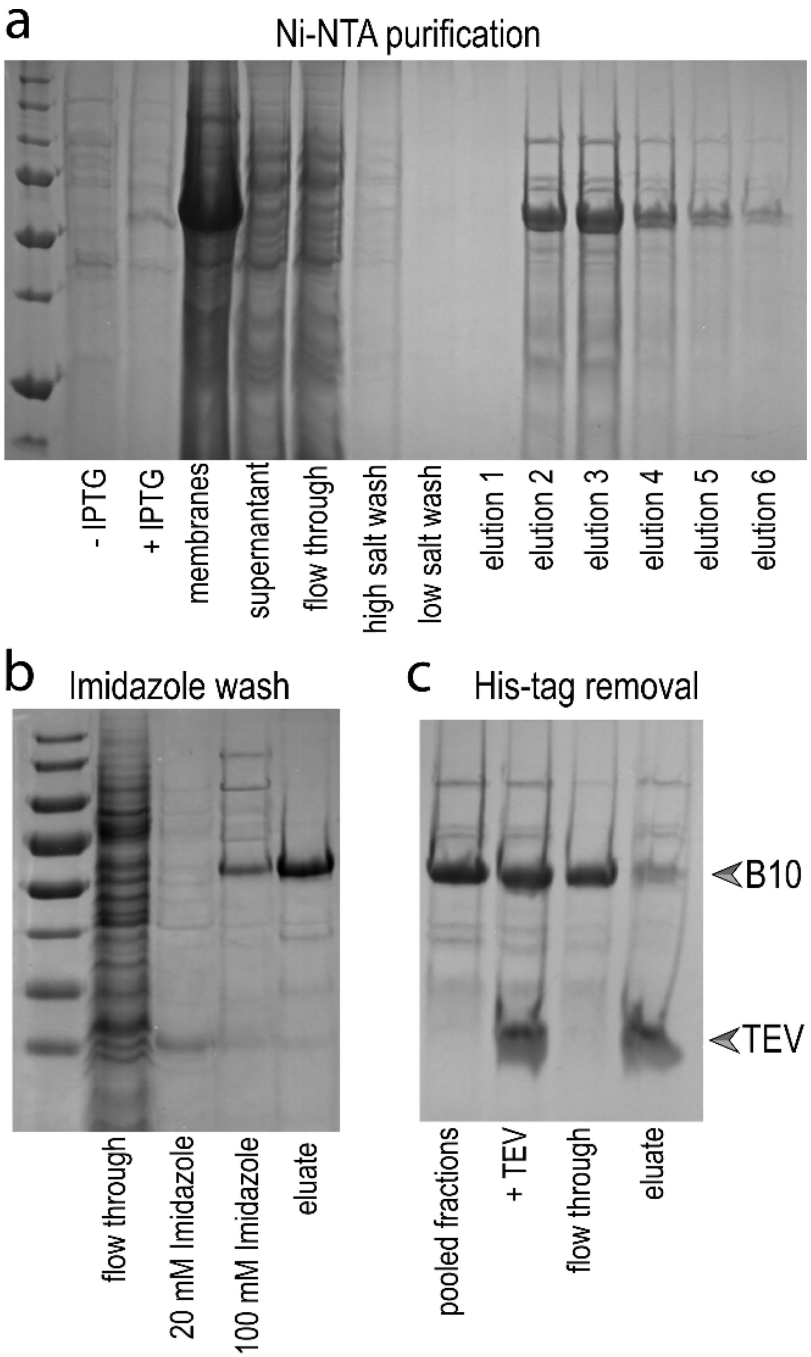

Figure 2.

Coomassie-stained gels of samples during purification steps. a, Affinity chromatography purification of N-terminus His-tagged ABCB10 expressed in BL21 cells. The gel shows, from left to right: confirmation of protein expression in cells after induction with IPTG, the membrane fraction, supernatant after high speed centrifugation of membranes solubilized with detergent, the flow through after incubation with Ni-NTA, wash with high and low salt buffer (1M and 0.3 M NaCl, respectively), and the elution fractions. b, Effect of high imidazole wash: column was washed with buffer containing 20 mM and 100 mM imidazole

before elution. c, TEV protease was added to pooled fractions for His-tag removal. The flow-through after incubation of this proteolysis reaction with Ni-NTA contains purified ABCB10. TEV, contaminants, and some ABCB10 that were bound to Ni-NTA can be observed in the eluate (with 300 mM imidazole). Arrowheads point to the positions corresponding to ABCB10 and TEV.

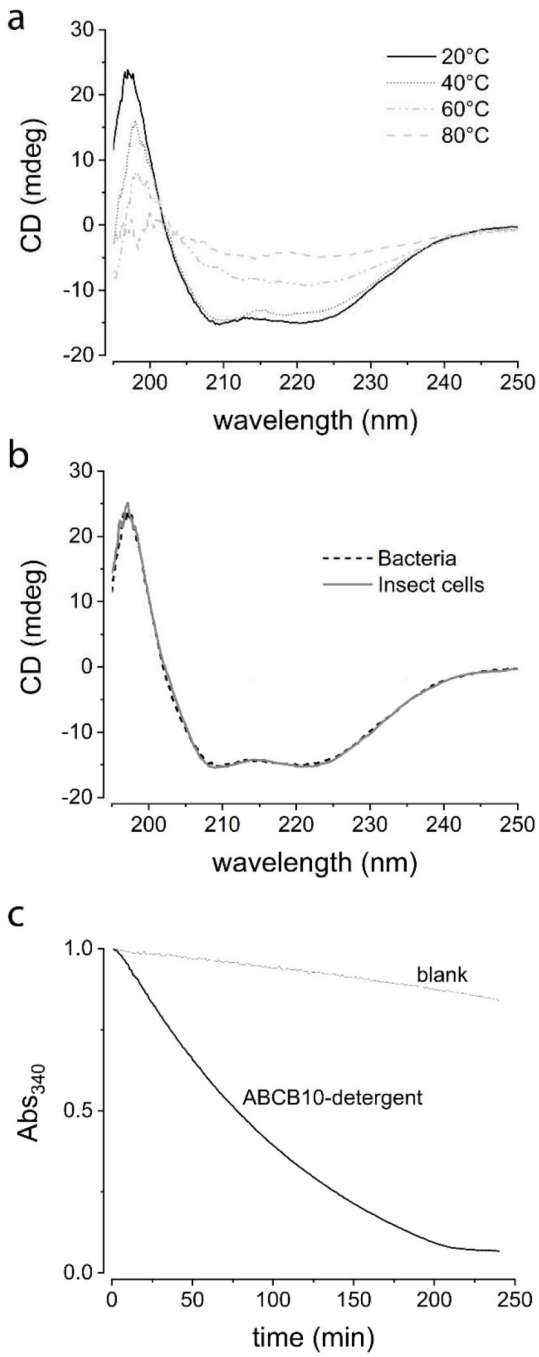

Fig. 3.

Characterization of purified ABCB10 in detergent micelles. a, Circular dichroism spectra of ABCB10 purified from bacteria, at different temperature. b, Circular dichroism spectra of ABCB10 purified either from insect cells or bacteria. c, Coupled-enzyme ATPase assay. The decreased NADH absorbance over time is coupled to ATP hydrolysis by ABCB10.

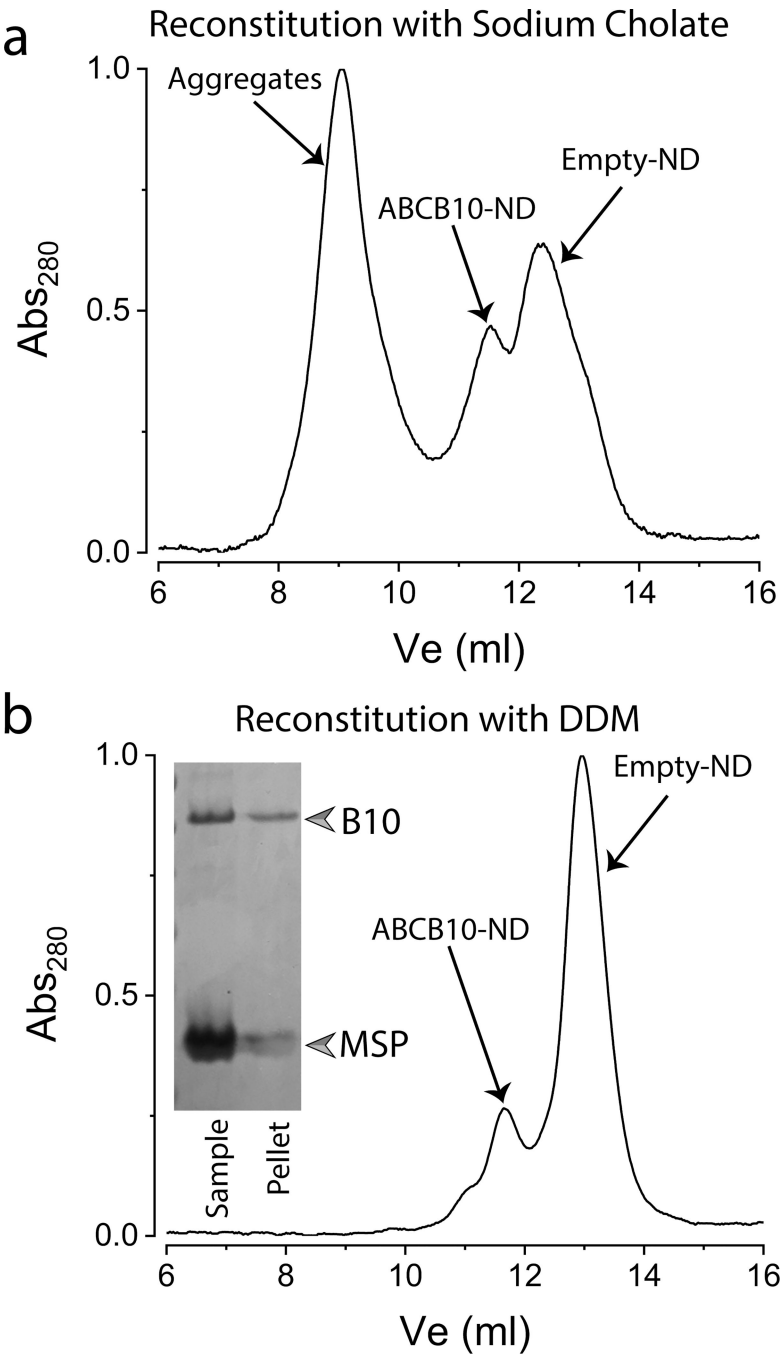

Fig. 4.

Size-exclusion profiles of ABCB10 reconstituted in lipid nanodiscs. a, Reconstitution using sodium cholate led to the appearance of protein aggregates. b, Reconstitution using DDM instead of sodium cholate led to formation of larger aggregates that can be removed by centrifugation of the sample, as observed in the Coomassie stained gel (inset). ABCB10-ND and Empty-ND are ABCB10-containing and empty nanodiscs, respectively. Arrowheads point to the position of ABCB10 and the membrane scaffold protein (MSP).

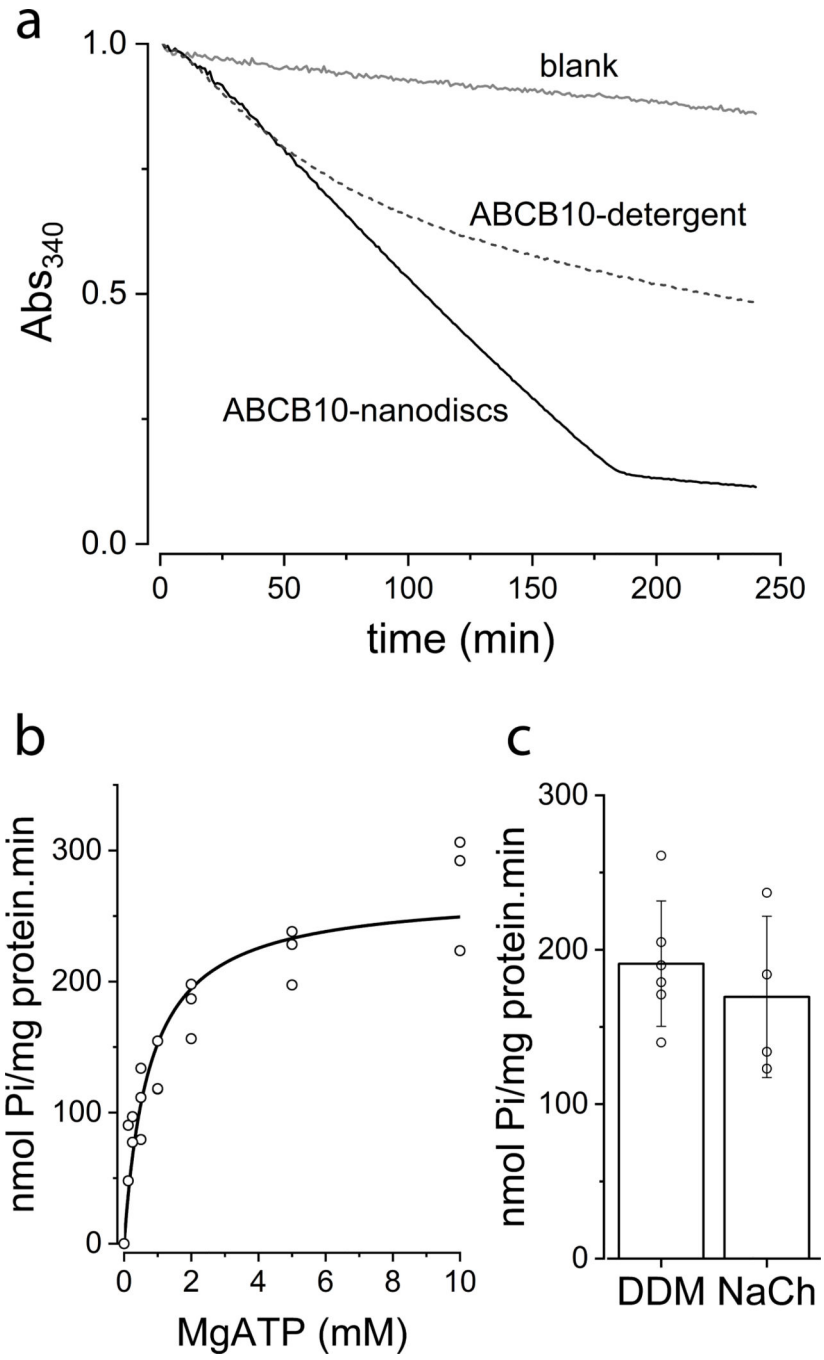

Fig. 5.

Characterization of ABCB10 reconstituted in lipid nanodiscs. a, Coupled-enzyme ATPase assay. The linear decrease on NADH absorbance over time indicates stability of the transporter in nanodiscs compared to detergent. b, ATP hydrolysis activity of ABCB10 (empty circles) fitted to Hill function (line) show a V_{max} of 268 ± 21 nmol Pi/mg protein*min and a K_m of 0.75 ± 0.2 mM (n = 3 independent experiments). c, ATPase activity

of ABCB10 reconstituted in nanodiscs using either DDM or sodium cholate (NaCh). A two-sample t-test indicated not difference between the samples ($p = 0.05$).