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Characterization and functional analysis of the nucleotide binding fold in human peroxisomal ATP binding cassette transporters

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Abstract The 70-kDa peroxisomal membrane protein (PMP70) and the adrenoleukodystrophy protein (ALDP) are half ATP binding cassette (ABC) transporters in the peroxisome membrane. Mutations in the *ALD* gene encoding ALDP result in the X-linked neurodegenerative disorder adrenoleukodystrophy. Plausible models exist to show a role for ATP hydrolysis in peroxisomal ABC transporter functions. Here, we describe the first measurements of the rate of ATP binding and hydrolysis by purified nucleotide binding fold (NBF) fusion proteins of PMP70 and ALDP. Both proteins act as an ATP specific binding subunit releasing ADP after ATP hydrolysis; they did not exhibit GTPase activity. Mutations in conserved residues of the nucleotidases (PMP70: G478R, S572I; ALDP: G512S, S606L) altered ATPase activity. Furthermore, our results indicate that these mutations do not influence homodimerization or heterodimerization of ALDP or PMP70. The study provides evidence that peroxisomal ABC transporters utilize ATP to become a functional transporter.

Key words: ATP binding cassette transporter; Nucleotide binding fold; Peroxisome membrane; X-linked adrenoleukodystrophy; Adrenoleukodystrophy protein; 70-kDa peroxisomal membrane protein

1. Introduction

The ATP binding cassette (ABC) transporter superfamily consists of membrane proteins involved in the transport of a variety of molecules across biological membranes [1,2]. ABC transporters are composed of two homologous halves, each containing a membrane-spanning region with multiple transmembrane segments and a nucleotide binding domain. Eukaryotic ABC transporters are found either as complete transporters or as half transporters, which dimerize to form an active transporter. The multiple drug resistance transporter, MDR, and the cystic fibrosis transmembrane regulator, CFTR, are examples of the former while the TAP1 and TAP2 proteins are examples of the latter [3–6]. Four half ABC transporter proteins have been identified in the human peroxisome membrane: the 70-kDa peroxisomal membrane protein (PMP70), the adrenoleukodystrophy protein (ALDP), the PMP70-related protein (P70R) and the ALDP-related protein (ALDR) [7–10].

Mutations in the *ALD* gene encoding ALDP cause X-linked ALD (X-ALD), the most common inherited peroxisomal disorder characterized by abnormal accumulation of saturated, very long chain fatty acids predominantly in brain white matter, adrenal cortex and testis [11–14]. The biochemical defect is localized to the level of lignoceroyl-CoA synthesis, a step in the peroxisomal β-oxidation of very long chain fatty acids. The exact functions of ALDP and the other peroxisomal ABC transporters as well as their interactions are poorly understood. Pxa1p and Pxa2p are yeast genes that encode homologs of peroxisomal half ABC transporters. Evidence from *Pxa1* and *Pxa2* mutants suggests that peroxisomal ABC transporters are involved in the import of fatty acids or fatty acyl-CoAs for the peroxisomal β-oxidation [15–17]. The nucleotide binding fold (NBF) motifs Walker A, Walker B and 19-mer are conserved sequences in the carboxyl-terminal half of ABC-transporters [18]. Alignment of prokaryotic and eukaryotic NBFs reveals several highly conserved residues. The *ALD* gene mutations of patients with X-ALD have a tendency to cluster in and close to the NBF [14]. To study the role of the NBF in ABC transporters for peroxisome function we generated wild type and mutant constructs of ALDP and PMP70. For the mutant constructs we selected X-ALD patient mutations in highly conserved residues in the Walker A and 19-mer region of the NBF of ALDP (G512S and S606L) and the corresponding PMP70 mutations (G478R and S572I). We overexpressed wild type and mutant proteins in fusion with the maltose binding protein (MBP) and analyzed nucleotide binding, ATPase and GTPase activities. We also used this expression system to assess the possible role of the NBF in protein–protein interaction.

2. Materials and methods

2.1. Materials

We obtained reagents from the following sources: synthetic oligonucleotides (MWG Biotech); pMALc2 expression vector, purification system, T4 DNA ligase, vent DNA polymerase, restriction enzyme *Xmn*I, factor Xa, amylose resin (New England Biolabs); DH5α, JM109 and TOPP I cells (Stratagene); dye terminator cycle sequencing kit (Applied Biosystems); 8-azido-[γ^{32}P]ATP and 8-azido-[γ^{32}P]GTP (ICN Biochemicals); molecular size markers (Bio-Rad, Gibco); ECL kit for Western blotting (Amersham); polyclonal rabbit anti-rat PMP70 (W. Just, Center of Biochemistry, University of Hei-

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Abbreviations: ABC, ATP binding cassette; ALDP, adrenoleukodystrophy protein; ALDR, ALDP-related protein; MBP, maltose binding protein; PMP70, 70-kDa peroxisomal membrane protein; P70R, PMP70-related protein; NBF, nucleotide binding fold; X-ALD, X-linked adrenoleukodystrophy; β-gal, α-subunit of β-galactosidase

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delberg, Heidelberg, Germany), monoclonal mouse anti-human ALDP NBF antibody (Euromedex); pcDNA3.1 plasmid (Invitrogen); ^{35}S-methionine (Amersham); Superdex 200 HR 10/30 column (Pharmacia); TNT-coupled reticulocyte lysate system (Promega). All other reagents were obtained from Boehringer or Sigma.

2.2. NBF: wild type and mutant construction

We amplified the NBF of PMP70 (amino acids (AA) 445–634) and ALDP (AA 478–667) by using human cDNA as a template. Thirty cycles were performed (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) followed by a final extension of 3 min at 72°C. The specific primers used for PMP70 are 5′-GGAATTCCATGTTCCTTTAG-CAACGCCA-3′ (primer I with *Eco*RI restriction site) and 5′-GCTCTAGACTATCACTCATGATGTTTCCAAAGAGA-3′ (primer II with *Xba*I restriction site and stop codon). The specific primers used for ALDP are 5′-AACATCCCCATCGTCACGC-3′ (primer I with blunt end) and 5′-GGAATTCAGTGGTATTTCCACAGG-GAG-3′ (primer II with *Eco*RI restriction site and stop codon). The identity of all constructs was confirmed by sequencing.

All site-directed mutagenesis was carried out according to the megaprimer method as described by Sarkar and Sommer [19] by using the following oligonucleotides:

Protein	Synthetic oligonucleotide	Mutation
ALDP	5′-CCCCAATGGCTGCAGCAAGAGCTCCC-3′ 5′-GGATCCGGACAGGGAGCTCTTGCTGCAGC-3′	G512S
ALDP	5′-ACTGGAAGGACGTCCTGTTGGG-3′ 5′-CGCCACCCAACAGGACGTCCTTCC-3′	S606L
PMP70	5′-GGCTGCAGAAAGAGTTCACTTTTCCG-3′ 5′-GGCCATAATTCACCAAGAACACGGAAA AGTGAACTCTTTCTG-3′	G478R
PMP70	5′-GACGTACTCATTGGTGGAG-3′ 5′-CCACCAATGAGTACGTCCATCCAATCC-3′	S572I

We subcloned an *Eco*RI–*Xba*I DNA fragment of the PMP70 NBF constructs and an *Xmn*I–*Eco*RI DNA fragment of the ALDP NBF constructs into the polylinker of the pMALc2 vector. By this strategy the NBF fragments were inserted in frame into the carboxyl-terminal coding region of the MBP cDNA. Both strands of the inserted fragments and the vector cloning sites were sequenced to confirm the identity of all constructs.

2.3. Transformation in JM109 and TOPP I competent cells

Isolated plasmid DNA was transformed using competent *Escherichia coli* JM109 cells for the PMP70 NBF constructs and *E. coli* TOPP I cells for the ALDP NBF constructs according to standard procedures.

2.4. Overexpression and purification of pMALc2 NBF fusion proteins

E. coli JM109 cells or TOPP I cells harboring the appropriate recombinant plasmid were grown at 37°C in LB medium with 200 μg ampicillin/ml and 12 mM glucose. At a cell density of 0.5 (OD_{600}) protein expression was induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h. Cells were harvested by centrifugation at 4000×*g* at 4°C for 20 min, resuspended and washed once with ice cold PBS. All following purification steps were carried out at 4°C. Cells were resuspended in 10 ml lysis buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25 ml protease inhibitor) per gram wet weight and lysed by ultrasound sonification with short pulses in an ice bath for 2 min. The lysate was diluted 1:4 with 1×column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 0,1% Triton X-100) and centrifuged at 10 000×*g* for 20 min. The supernatant was applied to a 12-ml amylose column equilibrated with column buffer. The column was first washed extensively with eight column volumes of column buffer. The fusion proteins were then eluted with column buffer containing 10 mM maltose. The eluted fractions were monitored by their absorption at 280 nm and subsequently analyzed by SDS–PAGE as described by Laemmli [20] and by Western blotting using a polyclonal rabbit anti-rat PMP70 antibody, a monoclonal mouse anti-human ALDP NBF antibody or a monoclonal mouse anti-MBP antibody. Protein concentrations were determined by the method of Lowry [21].

2.5. Photoaffinity labeling with 8-azido-[γ^{32}P]ATP and 8-azido-[γ^{32}P]GTP

2–4 μg of fusion protein was incubated in reaction buffer containing 2 mM $MnCl_2$, 10 mM $MgCl_2$ in the presence of either 3.5 μM 8-azido-[γ^{32}P]ATP or 5.5 μM 8-azido-[γ^{32}P]GTP. Some samples also contained 50 mM ATP, AMP or GTP. All samples were incubated at 0°C for 20 min, exposed to UV light at 0°C for 5 min, boiled with SDS–PAGE loading buffer for 2 min and analyzed on SDS–PAGE. The gels were dried and subjected to autoradiography. Exposure time of autoradiography was 2 h for ATP labeling and 10 h for GTP labeling. The identity of the labeled bands was confirmed by Western blotting.

2.6. Coupled assay for ATPase or GTPase activity

A coupled ATP or GTP regenerating enzyme system was used following the method described by Norby [22]. 2–11 μM fusion protein was incubated in a total volume of 360 μl with 3 mM phosphoenolpyruvate, 2.4 mM NADH, 6.5 mM magnesium acetate, 34 mM KCl, 4.17 μg (1.9 U) lactate dehydrogenase and 12.5 μg (1.9 U) pyruvate kinase in 50 mM Tris–HCl, pH 7.5 at 25°C. Increasing amounts of ATP or GTP (2.8–2055 μM) were added and $\Delta A_{340}/\Delta t$ was monitored.

2.7. In vitro translation and ^{35}S-methionine labeling of peroxisomal ABC transporter proteins

For in vitro translation and labeling, full length ALDP and PMP70 were cloned into pcDNA3.1 plasmid. The proteins were transcribed in vitro from the T7-promoter of pcDNA3.1, translated and labeled with ^{35}S-methionine using a TNT-coupled reticulocyte lysate system following standard protocols.

2.8. In vitro interaction assay

The purified MBP fusion proteins were bound to amylose resin in column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) for 1 h at 4°C and washed three times in column buffer. For the in vitro interaction assay the MBP fusion proteins (1.5–2 μg protein bound to 5 μl matrix suspension) were incubated with 5 μl of in vitro translated protein in 100 μl binding buffer (50–500 mM NaCl, 50 mM potassium phosphate pH 7.4, 1 mM $MgCl_2$, 1–10% glycerol, 0.1% Tween 20, 1.5% bovine serum albumin (BSA)) for 2 h at 4°C. Samples were pelleted and washed four times in 1 ml binding buffer without BSA. Pellets were resuspended in SDS sample buffer and boiled for 5 min before being analyzed by SDS–PAGE according to standard protocols. To verify stability of the MBP fusion proteins and equal loading, gels were stained with Coomassie blue. The gels were dried and exposed to X-ray film for 24 h.

2.9. Gel filtration chromatography of purified NBF fusion proteins

Purified wild type ALDP or PMP70 NBF fusion proteins were centrifuged at 10 000×*g* for 10 min. 100 μl of the supernatant was applied to a 24-ml Superdex 200 HR 10/30 column equilibrated with column buffer, the column run was at a flow rate of 0.5 ml/min. The eluted fractions were monitored by their absorption at 280 nm and subsequently analyzed by SDS–PAGE. The molecular weight was determined by comparing the ratio of $V_{elution}/V_{void}$ for the eluted proteins with the ratio of $V_{elution}/V_{void}$ for protein standards.

3. Results

3.1. Characterization of the NBF protein motif

Fig. 1 shows alignment of NBF motifs from the peroxisomal family of ABC transporters. These proteins exhibit the highest similarity in the Walker A and 19-mer region. We mutated the central glycine in the Walker A motif of PMP70 and ALDP making the evolutionary severe substitutions G478R and G512S. Additionally, we changed the conserved serine in the 19-mer motif of PMP70 and ALDP to isoleucine (S572I) and leucine (S606L), respectively.

3.2. Overexpression and purification of fusion proteins

The NBF of PMP70 and ALDP was expressed in fusion with the MBP in *E. coli*. The α-subunit of β-galactosidase (β-

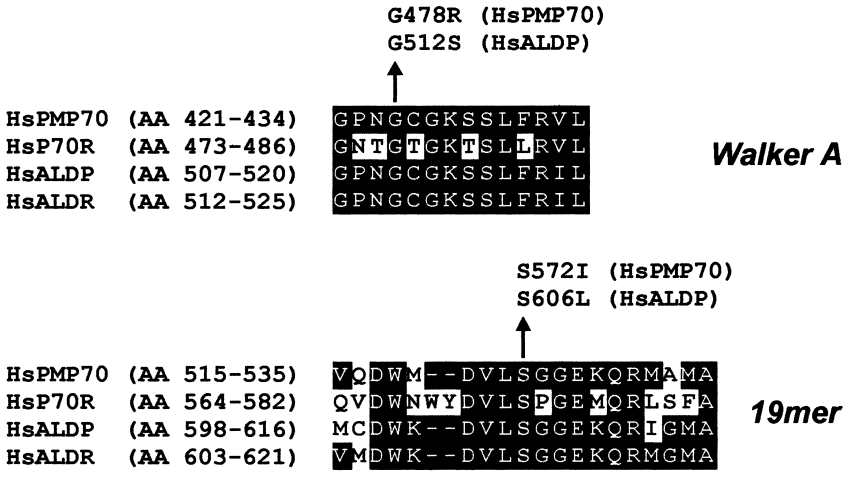

Fig. 1. Alignment of NBF motifs and position of mutations. Listed human peroxisomal ABC transporters from top to bottom are HsPMP70, HsP70R, HsALDP and HsALDR. AA present in a plurality of these sequences are boxed in black. Missense mutations produced are indicated above. G512S and S606L cause X-ALD.

gal) in fusion with the MBP was used as a control. Overexpression of the fusion proteins under control of the tac promoter after induction with IPTG revealed highest expression levels by using *E. coli* TOPPI cells for ALDP constructs and *E. coli* JM109 cells for PMP70 constructs and β-gal. In contrast, overexpression of these fusion proteins in *E. coli* DH5α cells resulted in negligible protein amounts. Fig. 2 shows that β-gal and the NBF of PMP70 are strikingly overexpressed upon induction and constitute a main component of the bacterial cell lysate. Protein expression levels for the NBF of ALDP are significantly lower when compared with PMP70. Nevertheless, induction with IPTG is also evident for ALDP when the induced expression level is compared with the uninduced cell lysate. The expression levels of the mutant PMP70 and ALDP fusion proteins were equal to the corresponding wild type protein levels (data not shown). Most of the overexpressed fusion proteins are soluble and have the expected molecular size of 64 kDa (42.7 kDa for MBP+21.4 kDa for ALDP NBF; 42.7 kDa for MBP+21.7 kDa for PMP70 NBF). As the MBP exhibits a high affinity for amylose, the purification of the fusion proteins by amylose column affinity chromatography was very effective. A yield of 5–10 mg or 10–20 mg of purified protein per liter of cell culture was achieved for ALDP and PMP70 constructs, respectively. A variety of prominent protein bands smaller than 64 kDa were present in the purified ALDP NBF fusion proteins. These bands were shown to be truncated fusion proteins by immunoblotting and amino-terminal sequence analyses. The purity of the NBF fusion proteins was estimated from SDS–PAGE to be $>95\%$ pure (Fig. 3).

We further characterized all fusion proteins by immunoblotting using antiserum to MBP, PMP70 and the NBF of ALDP. The pattern of the MBP immunoblot (Fig. 4A) was identical to the one of the Coomassie-blue-stained SDS–PAGE gel (Fig. 3). All bands smaller than 64 kDa corresponded to truncated fusion proteins. The ALDP immunoblot (Fig. 4B) as well as the PMP70 immunoblot (Fig. 4C) revealed bands of the expected molecular size of 64 kDa indicating that the NBFs of ALDP and PMP70 are inserted in frame and that the integrity of the NBF was maintained during purification. The antiserum to the ALDP NBF did not show any cross reactivity to the PMP70 NBF.

3.3. Nucleotide binding function of ALDP and PMP70

The purified NBF fusion proteins were photoaffinity-labeled with 8-azido-[γ^{32}P]ATP or 8-azido-[γ^{32}P]GTP. BSA, an ATP and GTP binding protein, was used as a control [23,24]. The corresponding 64-kDa protein band of the photolabeled ALDP or PMP70 NBF domain was identified by immunoblotting with specific antibodies. Fig. 5 shows that the purified NBF fusion proteins are labeled by 8-azido-[γ^{32}P]ATP. There was no major difference in the level of ATP binding between the ALDP and PMP70 NBF domains. In contrast to the striking ATP binding, there was only a weak 8-azido-[γ^{32}P]GTP band for ALDP and PMP70 NBF (data not shown). The exposure time for getting ATP- and GTP-labeled bands of the same intensity is five times longer for GTP than for ATP indicating that ATP is the predominant substrate for

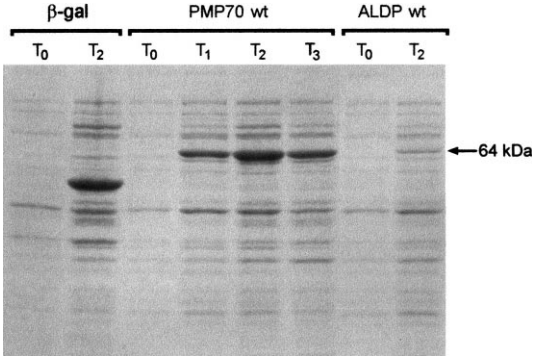

Fig. 2. Overexpressed NBF fusion proteins. 75 μl of bacterial cell lysate of *E. coli* JM109 cells containing recombinant plasmid of β-gal or PMP70 wild type (wt) and *E. coli* TOPP I cells containing the recombinant plasmid of ALDP wild type (wt) were solubilized in SDS sample buffer and separated by SDS–PAGE. The lanes designated as T_0 correspond to uninduced cells, the lanes marked as T_1, T_2 and T_3 to cell lysate 1, 2 and 3 h after IPTG induction, respectively. Both NBF proteins exhibit an approximate molecular mass of 64 kDa.

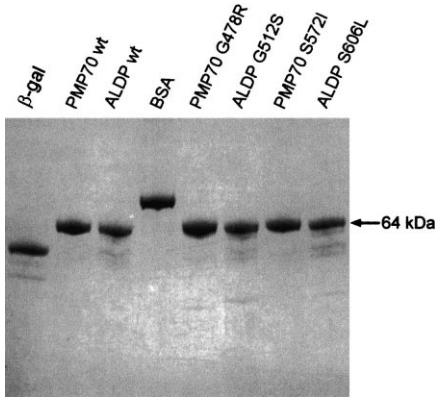

Fig. 3. Overexpressed and purified wild type and mutant NBF fusion proteins. 3 μg of each fusion protein purified by amylose affinity chromatography were subjected to SDS–PAGE. The fusion proteins used are β-gal, wild type PMP70 and ALDP, PMP70 and ALDP mutants; BSA was used as a control. The wild type and mutant NBF fusion proteins have an approximate molecular mass of 64 kDa.

ALDP and PMP70. BSA was labeled by 8-azido-[γ^{32}P]ATP and also by 8-azido-[γ^{32}P]GTP. In contrast to previous studies [25], we found a very weak ATP and GTP binding of β-gal in fusion with MBP. Labeling of all fusion proteins could be inhibited if the sample was preincubated with non-radioactive nucleotides prior to photoaffinity labeling.

3.4. ATPase and GTPase activity of ALDP and PMP70

For investigating the intrinsic ATPase and GTPase activity of the purified ALDP and PMP70 fusion proteins a coupled ATP or GTP regenerating enzyme system was used. The assay monitors the formation of ADP coupled to the pyruvate kinase and lactic dehydrogenase reactions by photometric analysis. We did not detect any ATPase activity neither for the β-gal fusion protein nor for MBP. Since the MBP is not a nucleotide binding protein, we measured ATPase and GTPase activities of PMP70 and ALDP in fusion with the MBP and did not cleave the NBF protein motif before analysis. The wild type and mutant ALDP and PMP70 NBF fusion proteins were able to hydrolyze ATP (Fig. 6). The hydrolysis rate of the fusion proteins was found to be linear to the different protein concentrations examined. We obtained a typical Michaelis–Menten curve by plotting ATPase activity versus ATP concentration. Results from different assay procedures are summarized in Table 1. The S606L and G478R mutants have a decreased ATP binding affinity while the G512S and S572I mutants decrease the maximum velocity of ATPase activity. Using an analogous assay for GTPase activity measurement we could not detect any GTPase activity for ALDP and PMP70 NBF fusion proteins.

3.5. NBF domain interactions of ALDP and PMP70

To determine if the NBF is involved in dimerization, we incubated the purified NBF fusion proteins with the corresponding in vitro translated and labeled full length ABC transporter protein. We analyzed these samples on SDS–PAGE. When testing the NBF of wild type ALDP and wild type PMP70 as well as the NBF of the mutant ALDP (G512S) against full length ALDP or full length PMP70, only background levels were detected (data not shown). These results indicate that the NBF domain is not necessary and sufficient for homodimerization or heterodimerization of ALDP and PMP70.

We also analyzed the dimerization capability of wild type NBF fusion proteins by gel filtration chromatography. The elution profiles of both NBF fusion proteins were comparable and showed a single peak at 64 kDa. We did not detect a peak in the molecular weight region of approximately 130 kDa, the region for heterodimerized or homodimerized NBF fusion proteins. These results confirmed our previous results that there is no evidence for protein–protein interaction in the NBF of PMP70 and ALDP.

4. Discussion

ABC transporters are present in all cellular living organisms and are involved in the transport of a variety of substrates across membranes [1,2]. Mutations in genes encoding ABC transporters are responsible for genetic diseases including the *CFTR* gene for cystic fibrosis and the *ALD* gene for X-ALD [8,26,27]. Several recent studies suggest that the carboxyl-terminal halves of these proteins including the NBF play an important role in protein function. Missense mutations in the NBF conserved sequences Walker A, Walker B and 19-mer influence substrate specificity and nucleotide binding [26,27].

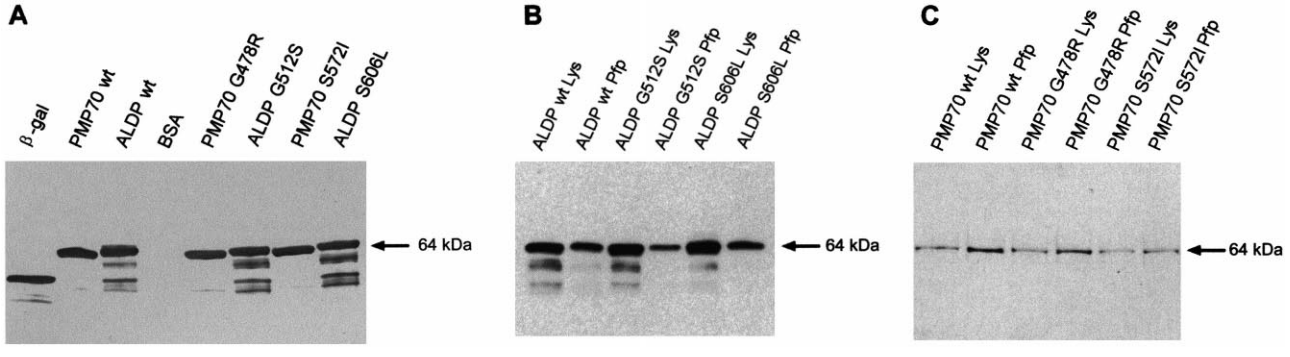

Fig. 4. Immunoblot of NBF fusion proteins. 1 μg of lysate or purified fusion proteins were analyzed including β-gal, wild type PMP70 and ALDP, BSA, PMP70 and ALDP mutants. Rabbit antiserum to either MBP (A) or PMP70 (C) and mouse antiserum to the NBF of ALDP (B) were used. The wild type and mutant NBF fusion proteins have an approximate molecular mass of 64 kDa. Lys, lysate; Pfp, purified fusion protein.

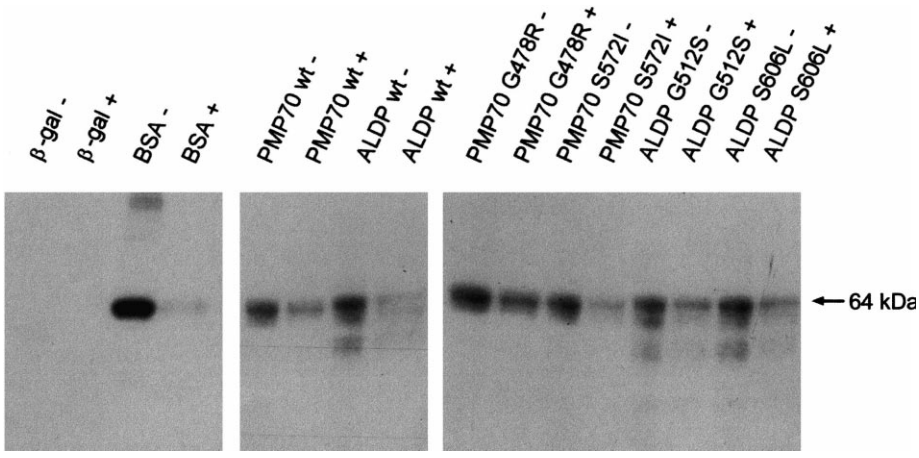

Fig. 5. Photolabeling of NBF fusion proteins. Purified fusion proteins were photolabeled with 8-azido-[γ^{32}P]ATP in the presence (+) or absence (−) of ATP. 3 μg of protein were analyzed including BSA, β-gal, wild type PMP70 and ALDP, PMP70 and ALDP mutants.

Liu and co-workers have shown that homo- as well as heterodimerization occurs between the carboxyl-terminal halves of ALDP, ALDRP and PMP70 [28]. We utilized recombinant polypeptide models to analyze different possible functions of the NBF in peroxisomal ABC transporters, namely ATP binding, ATPase activity and protein–protein interactions.

Contreras and co-workers [29] revealed that the NBF of ALDP is on the cytoplasmic surface of peroxisomal membranes and able to bind ATP. Moreover, we found that the NBF of ALDP and PMP70 can bind ATP and function as an ATPase. Our studies also provide evidence that ALDP and PMP70 are also GTP binding proteins. To further characterize the NBF, we applied site-directed mutagenesis in the genes encoding ALDP and PMP70. We found that mutations in conserved residues in the Walker A and 19-mer region alter ATPase activity. The missense mutation G512S in the *ALD* gene causes X-ALD and reduces considerable ATPase activity in our recombinant polypeptide models. This observation suggests that the peroxisomal β-oxidation process is tightly coupled to ATPase activity and that ATPase dysfunction leads to the accumulation of VLCFA in X-ALD patients. In contrast, the X-ALD patient mutation S606L alters ATP binding affinity but has no effect on the overall ATPase activity. Because the carboxyl-terminal halves of ALDP, PMP70 and ALDR are involved in protein dimerization and interactions with other protein motifs like the transmembrane domain [28], it is possible that the S606L missense mutation in ALDP exerts its disease causing effect in this manner. However, our results indicate that the NBF domain is not necessary and sufficient for homodimerization or heterodimerization of ALDP and PMP70. Our results are in accordance with the one by Liu and co-workers who by using deletion constructs of the carboxyl-terminal part of ALDP showed that the critical domain for dimerization is most likely carboxyl-terminal of the NBF [28].

Applying the pMALc2 expression system, we obtained purified soluble preparations of wild type and mutant NBFs of peroxisomal ABC transporters. The finding that the resultant fusion proteins bind amylose, ATP and GTP and that ATPase activity is preserved demonstrates that within the fusion complex both the NBF and MBP have folded correctly to allow formation of their respective substrate binding sites. The wild type and mutant NBF fusion proteins were shown to be virtually indistinguishable in their properties to bind ATP and GTP. Overexpression of these fusion proteins in large amounts and rapid purification in a single step provides an excellent basis for future attempts to form crystals for X-ray diffraction structural analyses of peroxisomal ABC transporters.

The specific ATPase activity of the isolated NBF of human peroxisomal ABC transporters is in the same order of magnitude than those of other ABC transporters including human CFTR [30,31] and MDR [32]. ATP affinity is 10–200 times higher when compared to CFTR and MDR, respectively. Nevertheless, the kinetic data obtained for isolated NBF domains do not represent the nucleotide binding and hydrolysis rate of complete native transporters. Previous studies on MDR suggest that the ATPase activity of the native protein

Table 1
Kinetic parameters of ATPase activity in wild type and mutant ALDP and PMP70 NBF fusion proteins

Fusion protein	K_M (μM)	V_{max} (nmol/μmol NBF/min)	Specific activity (10^{-3} U/mg)
ALDP (wild type)	11.5 ± 0.97	641.9 ± 10.7	10.0 ± 0.17
ALDP (G512S)	17.9 ± 1.23	279.3 ± 4.2	4.4 ± 0.06
ALDP (S606L)	45.6 ± 2.40	666.0 ± 8.7	10.4 ± 0.14
PMP70 (wild type)	8.2 ± 0.52	580.8 ± 6.7	9.0 ± 0.10
PMP70 (G478R)	161.8 ± 34.40	641.2 ± 28.2	10.0 ± 0.44
PMP70 (S572I)	9.9 ± 0.82	298.1 ± 4.7	4.6 ± 0.07

The kinetic data of all fusion proteins are mean values and standard deviations of 15–20 measurements at various protein concentrations using at least two distinct protein preparations.

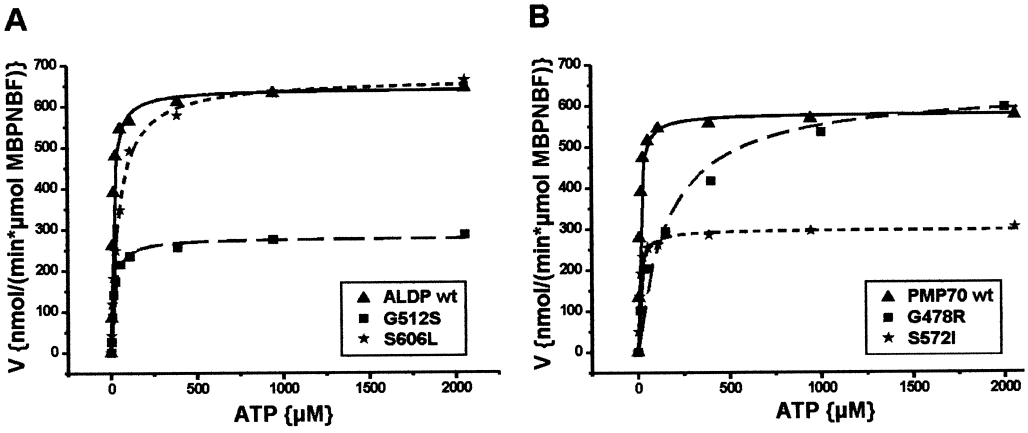

Fig. 6. Specific ATPase activity in NBF fusion proteins. Enzymatic activity of wild type and mutant ALDP (A) and PMP70 (B) was monitored by following the formation of ADP in a coupled spectrophotometric assay. The data points are the mean values of 15–20 measurements using at least two different protein preparations. Michaelis–Menten plots were calculated by hyperbolic curve fitting of mean values.

is two orders of magnitude higher when compared with isolated NBF. The activity is also substrate specific and increased by protein phosphorylation [32–36]. Regarding X-ALD patient mutations in the NBF, the effect of these disease causing mutations on ATPase activity or ATP binding affinity is most likely several orders of magnitude higher in the native protein when compared to our measurements in NBF fusion proteins. To further characterize the molecular functions and interactions of human peroxisomal ABC transporters it will be necessary to establish in vitro models of purified full length recombinant proteins with retention of the biochemical and physiological characteristics of an ABC transporter associated in its native peroxisomal membrane form.

In summary, our findings give the first experimental evidence that the NBF of two peroxisomal ABC transporters, ALDP and PMP70, can act as an ATP binding subunit that releases ADP after ATP hydrolysis. Missense mutations in the NBF cause ATPase dysfunction, and mutations in corresponding residues of ALDP cause the human peroxisomal disorder X-ALD. The NBF seems not to be directly involved in homodimerization and heterodimerization of these ABC transporters. Our studies indicate that recombinant polypeptide models are a useful system for elucidating the pathogenesis of ALD.

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