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ORIGINAL ARTICLE

Carla P. Guimarães · Clara Sá-Miranda
Jorge E. Azevedo

Probing substrate-induced conformational alterations in adrenoleukodystrophy protein by proteolysis

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Abstract The adrenoleukodystrophy protein (ALDP) is a half-ABC (ATP-binding cassette) transporter localized in the peroxisomal membrane. Dysfunction of this protein is the cause of the human genetic disorder X-linked adrenoleukodystrophy (X-ALD), which is characterized by accumulation of saturated, very-long-chain fatty acids (VLCFAs). This observation suggests that ALDP is involved in the metabolism of these compounds. Whether ALDP transports VLCFAs or their derivatives across the peroxisomal membrane or some cofactors essential for the efficient peroxisomal β-oxidation of these fatty acids is still unknown. In this work, we used a protease-based approach to search for substrate-induced conformational alterations on ALDP. Our results suggest that ALDP is directly involved in the transport of long- and very-long-chain acyl-CoAs across the peroxisomal membrane.

Keywords ATP-binding cassette transporters · ABCD1 transporter · Lipid transport · VLCFA metabolism · X-linked adrenoleukodystrophy · ALDP function

Abbreviations ABC: ATP-binding cassette · ALDP: Adrenoleukodystrophy protein · VLCFA: Saturated very-long-chain fatty acid · X-ALD: X-linked adrenoleukodystrophy

Introduction

X-linked adrenoleukodystrophy (X-ALD) is the most common inherited peroxisomal disorder with a minimum incidence of 1:21,000 in the male population (Bezman et al. 2001). It is a progressive neurodegenerative disease affecting mainly the nervous system white matter and the adrenal cortex. The clinical presentation is extremely variable and, frequently, different phenotypes occur in the same family. Common to all the X-ALD phenotypes, however, is the accumulation of saturated, unbranched, very-long-chain fatty acids (VLCFAs) in plasma and tissues of the patients (Moser et al. 2001).

The gene responsible for X-ALD (ABCD1 gene) was identified in 1993 (Mosser et al. 1993). It encodes a half-ATP-binding cassette (ABC) transporter, the so-called ALDP (or ABCD1). Half-ABC transporters are homodimeric or heterodimeric protein assemblies that couple ATP hydrolysis to the transport of substrates across biological membranes (Dean et al. 2001). Data suggesting that ALDP from mouse liver is a homodimeric protein were recently described [see Discussion in (Guimaraes et al. 2004)].

Several hypotheses have been forwarded to explain how dysfunction of ALDP leads to accumulation of VLCFAs in X-ALD [e.g., (McGuinness et al. 2003; Mosser et al. 1993)]. However, the possibility that ALDP may play a direct role in the metabolism of these fatty acids is still most plausible [e.g., (Aubourg and Dubois-Dalcq 2000; Braiterman et al. 1998; Dubois-Dalcq et al. 1999; Kemp et al. 2001)]. The observations supporting this hypothesis are not limited to the fact that β-oxidation of VLCFAs is a peroxisomal event (Lazarow and De Duve 1976) and that ALDP is a peroxisomal membrane protein (Mosser et al. 1994). Indeed, a variety of genetic and biochemical studies on ALDP-related peroxisomal ABC transporters from other organisms have led to the same conclusion. For example, disruption of Pxa1p and/or Pxa2p, the only two peroxisomal ABC transporters known in *Saccharomyces cerevisiae*, results in impaired growth of these mutants on long-chain fatty acids as a sole carbon source (Hettema et al. 1996; Shani and Valle 1996; Shani et al. 1995). Biochemical characterization of these

C. P. Guimarães · C. Sá-Miranda · J. E. Azevedo (✉)
UNILIPE, Instituto de Biologia Molecular e Celular,
Rua do Campo Alegre, 823, 4150-180 Porto, Portugal
E-mail: jazevedo@ibmc.up.pt
Tel.: +351-2-26074900
Fax: +351-2-26099157

C. P. Guimarães · J. E. Azevedo
Instituto de Ciências Biomédicas de Abel Salazar,
Porto, Portugal

proteins demonstrated their involvement in the transport of acyl-CoAs across the peroxisomal membrane (Hettema et al. 1996; Verleur et al. 1997). More recently, a similar role was proposed for the Comatose/Ped3 gene product of *Arabidopsis thaliana* (Footitt et al. 2002; Zolman et al. 2001). Obviously, all these observations do not prove that ALDP has a similar role in the metabolism of VLCFAs, but they do provide a ground of testable hypotheses.

If it is assumed that ALDP is directly involved in the β-oxidation of VLCFAs, and if we consider that the subcellular compartment where these fatty acids are activated to their CoA thioesters is presently unknown [for a review see (Moser et al. 2001)], then there are at least three different mechanisms leading to the same outcome: ALDP may be directly involved in the peroxisomal import of (1) free VLCFAs, (2) very-long-chain acyl-CoAs, or (3) a cofactor necessary for the β-oxidation of VLCFAs. In this work, we have addressed the first two possibilities. Our results suggest that ALDP is involved in the translocation of long- and very-long-chain acyl-CoAs across the peroxisomal membrane.

Materials and methods

Materials

Factor Xa protease was obtained from New England Biolabs (#P8010, lot 55). Acetyl-CoA and dodecanoyl-CoA were purchased from Roche and Fluka, respectively. Docosanoyl-CoA and tetracosanoyl-CoA were synthesized by American Radiolabeled Chemicals. All the other reagents were from Sigma.

Subcellular fractionation of placenta

A human placenta was obtained from the local maternity hospital. An informed written consent was granted. The tissue was chilled on ice immediately after birth and transported to the laboratory. The following procedures were carried out at 4°C: The placenta was washed with SEI buffer (0.25 M sucrose; 1 mM EDTA-NaOH, pH 7.4; and 5 mM imidazole-HCl, pH 7.4) and the adherent membranes were cut off. A portion of approximately 60 g was cut into small pieces and extensively washed in SEI buffer. The tissue was homogenized using a polytron (model PT10/35, Kinematica, Switzerland) in four volumes of SEI buffer supplemented with 0.1 mg/ml PMSF and a mammalian protease inhibitor cocktail (Sigma) at a 1:450 (v/v) final dilution. An organelle fraction was prepared by differential centrifugation. Briefly, the homogenate was centrifuged at 300× g for 10 min at 4°C in the SS-34 rotor (Sorvall, model RC-5B PLUS) and afterward at 3,200× g for 10 min. Finally, the organelle fraction was obtained by centrifugation at 20,000× g for 20 min and resuspended in SEI buffer. Aliquots (20–30 mg/ml) were frozen immediately in liquid nitrogen and stored at −70°C.

Protease treatment and extraction of membrane proteins

An aliquot of 0.45 mg of placenta organelle protein was incubated at 26°C for 1 h with 9 μg Factor Xa in 0.9 ml of buffer containing 0.22 M sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, and 10 mM $MgCl_2$. Endogenous proteases were inhibited with 0.1 mg/ml PMSF (added from a 50 mg/ml stock solution in ethanol) and 1:200 (v/v) mammalian protease inhibitor cocktail for 5 min on ice. Extraction of membranes with alkaline or low and high ionic strength solutions were performed essentially as described (Fujiki et al. 1982; Gouveia et al. 2000).

In vitro synthesis of radiolabeled ALDP and cleavage with Factor Xa

The cDNA encoding the human full-length ALDP transporter was amplified by RT-PCR using the F1 and F8 oligonucleotides exactly as described previously (Guimaraes et al. 2001) and cloned into the pGEM-T easy vector (Promega). Subsequently, the cDNA was inserted into the *Eco*RI site of pGEM-4 (Promega). Clones with ALDP cDNA in the correct orientation were selected for further in vitro transcription expriments with the Sp6 RNA polymerase. The plasmid was linearized with *Hin*dIII endonuclease and transcribed according to the manufacturer's instructions (Roche). ^{35}S-labeled ALDP was synthesized using the translation kit Reticulocyte Type II (Roche) in the presence of ^{35}S-methionine (specific activity >1,000 Ci/mmol; ICN Biomedicals).

One microliter of reticulocyte lysates containing ^{35}S-labeled ALDP was added to 60 μl of buffer containing 0.25 M sucrose, 40 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, and 0.2 M NaCl. Then, Factor Xa was added and the reaction extended over 1 h 30 min at 26°C.

Preparation of lipids

Stock solutions of acyl-CoA esters were prepared at a final concentration of 0.3 mM in $CHCl_3$:CH_3OH (1:1). These were dried under a stream of nitrogen and resuspended with α-cyclodextrin (10 mg/ml in 20 mM Tris-HCl, pH 8.0) in order to obtain 1 mM of final concentration lipid. The suspensions were sonicated for 1 h at room temperature in an ultrasonic water bath (Sonorex super RK255H, Bandelin). After checking the pH, single-use aliquots were stored at −70°C. It should be noted that all efforts to solubilize docosanoyl-CoA and tetracosanoyl-CoA in aqueous buffer (e.g., by increasing the ratio of α-cyclodextrin/lipid, increasing the solubilization temperature, extending the sonication time, or lowering the lipid concentration to half) were ineffective.

Suspensions of these two lipids were stored in aliquots at −70°C. In the experiment presented in Fig. 2c, dodecanoic and eicosanoic acids were added directly from a 6.5 mM stock solution in methanol. All the other samples received the same volume of methanol.

Factor Xa cleavage assay

The Factor Xa cleavage assays (60 µl final volume) were performed as follows: 45 µg of placenta organelle protein were incubated in cleavage buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20 µM oligomycin (prepared as 10 mM stock solution in ethanol), 0.22 M sucrose, 1 mM ouabain, and 0.1 % (v/v) Triton X-100) containing, where indicated, 10 mM nucleotides (added from 100 mM stock solutions, pH 7.5 adjusted with NaOH) and/or 200 µM lipids. After 10 min at 26°C, Factor Xa (2 µg) was added, and the incubation was continued for 45 min at the same temperature. The reaction was stopped with trichloroacetic acid (TCA), and the samples were analyzed by Western blotting.

The intrinsic Factor Xa protease activity, in the presence of dodecanoyl-CoA, eicosanoyl-CoA, ATP, ATPγS, dodecanoyl-CoA plus ATP or ATPγS, and eicosanoyl-CoA plus ATP or ATPγS, was assayed against a chromogenic substrate (Sigma F3301). Factor Xa protease (6 ng) was incubated with 10 mM nucleotides and/or 200 µM acyl-CoAs esters in a final volume of 120 µl cleavage buffer (see above) for 5 min at room temperature. The Factor Xa substrate (0.2 mM) was added, and the reaction was followed spectrometrically at 405 nm over 30 min using a kinetic microplate reader (Sunrise, Tecan, Switzerland). Under these conditions, hydrolysis of the Factor Xa substrate was linear over this period (data not shown).

Miscellaneous

Protein quantification, SDS-PAGE, and Western blotting were performed as described previously (Guimaraes et al. 2004). The anti-ALDP monoclonal antibody (clone 1D6; Euromedex) and the rabbit antihuman ALDP antibody (Watkins et al. 1995) (a kind gift of Prof. Paul Watkins, Kennedy Krieger Institute, Baltimore, USA) were used in the Western-blotting analysis at 1:1,000 (v/v) dilution. For detection, either chemiluminescent (Super Signal West Dura, Pierce) or chromogenic substrates (Biorad) were used. Densitometric analysis of Western blots was performed using the UN-SCAN-IT automated digitizing program.

Results and discussion

Rationale and principle of the method

ABC transporters are molecular pumps that couple ATP hydrolysis to the vectorial transport of molecules across a biological membrane. Although our knowledge on the mechanism by which this process occurs is still limited, data indicating that these proteins oscillate between different conformations during the catalytic cycle are frequent in the literature [for a review see (Jones and George 2004)]. The nature and amplitude of these conformational alterations is still a matter of debate. Nevertheless, at least in some cases, a simple protease-based assay can easily reveal such structural rearrangements (Julien and Gros 2000; Manciu et al. 2003; Wang et al. 1998). In this work, we have used this strategy in order to gather data on the type of substrate transported by ALDP. Our premise was that in the presence of the correct substrate, the conformation of ALDP would change and consequently a different susceptibility to proteolysis should be observed.

Thus, our first task was to search for a protease that could allow us to detect substrate-induced conformational alterations on ALDP. In a preliminary set of experiments, a human placenta organelle fraction (see Material and methods) was incubated in the presence of either nonesterified VLCFAs or the corresponding CoA esters and treated with increasing concentrations of several proteases (data not shown). The behavior of ALDP was monitored by Western blotting analysis using two antibodies directed to different domains of the protein (see below). From the several proteases tested, Factor Xa proved to be the most promising reagent for this analysis. The results obtained with this protease are described below.

Characterization of the proteolytic pattern of ALDP after Factor Xa cleavage

The characterization of the proteolytic profile of ALDP obtained after Factor Xa cleavage in the absence of exogenous substrates is presented in Fig. 1. After digestion, two different ALDP fragments were detected: one of these fragments displayed an apparent molecular mass of approximately 44 kDa upon SDS-PAGE and was recognized by the monoclonal antibody 1D6 produced against residues 279–482 of the protein (Mosser et al. 1994); the other fragment displayed 33 kDa and was detected by the polyclonal antibody directed to the last 18 carboxy-terminal amino acids of human ALDP (Watkins et al. 1995) (see Fig. 1a).

In order to characterize further these two ALDP fragments, their interaction with the peroxisomal membrane was studied. For this purpose, Factor Xa-treated organelles were subjected to alkaline extraction or were sonicated in the presence of low or high ionic strength buffer. Membrane fractions and extracted (soluble) proteins were then prepared by centrifugation and analyzed by Western blotting. As shown in Fig. 1b, the 44-kDa ALDP fragment was detected in all the membrane fractions, implying that this domain of ALDP is intrinsic to the membrane. In contrast, the 33-kDa fragment displayed the properties of an extrinsic membrane

protein since it is readily extracted from the membranes at alkaline pH but not by sonication of the organelles in the presence of low or high ionic strength saline buffer. These results could suggest that, at the concentration used in these assays, Factor Xa cleaves ALDP into two main fragments: one corresponding to the membrane-embedded N-terminal half of ALDP (the 44-kDa fragment) and the other comprising the C-terminal nucleotide-binding domain of the protein (the 33-kDa fragment). Indeed, data indicating the existence of a preferred (but not unique) Factor Xa cleavage site in the central region of ALDP was obtained when the ^{35}S-labeled in vitro synthesized ALDP was incubated in the presence of the protease. As shown in Fig. 1c, although several ALDP-derived polypeptides can be obtained by treatment with Factor Xa, two major proteolytic products migrating exactly as the 44-kDa and 33-kDa fragments described above were easily detected (bands a1 and a2, respectively).

It should be noted that the exact Factor Xa cleavage sites on ALDP cannot be located simply using primary structure-based considerations. It is well established that Factor Xa hydrolyses peptide bonds with high efficiency at the carboxyl side of arginine residues, particularly if these residues are preceded by a glycine residue. However, as shown recently, several other amino acid residues at the penultimate position of the cleavage site are also allowed (Harris et al. 2000).

Long- and very-long-chain acyl-CoAs increase the sensitivity of the N-terminal domain of ALDP to proteolysis by Factor Xa

As shown above, treatment of ALDP with Factor Xa results in the production of two major fragments. Virtually the same result was obtained when the protease assay was performed in cleavage buffer (see Materials and methods) in the presence of 200-μM reduced CoA, acetyl-CoA, octanoyl-CoA, or dodecanoyl-CoA (Fig. 2a; compare lanes 2–5 with lane 1). In sharp contrast, when the same concentration of hexadecanoyl-CoA, eicosanoyl-CoA, docosanoyl-CoA, or tetracosanoyl-CoA were used in these assays, the amount of the ALDP 44-kDa N-terminal fragment that resisted to the protease was highly decreased (Fig. 2a; compare lanes 6–9 with lanes 1–5). No significant variations in the amount of the 33-kDa ALDP C-terminal fragment were detected under the different conditions used in this work (see Fig. 2a; data not shown).

Densitometric analysis of the band corresponding to the 44-kDa ALDP fragment reveals that long- and very-long-chain acyl-CoAs decrease the amount of Factor-Xa-resistant fragment with different efficiencies: eicosanoyl-CoA > hexadecanoyl-CoA ~ docosanoyl-CoA > tetracosanoyl-CoA. While the relationship between the effects of eicosanoyl-CoA and hexadecanoyl-CoA is not surprising [the majority of hexadecanoyl-CoA is oxidized in mitochondria in vivo (Wanders 2004)], the

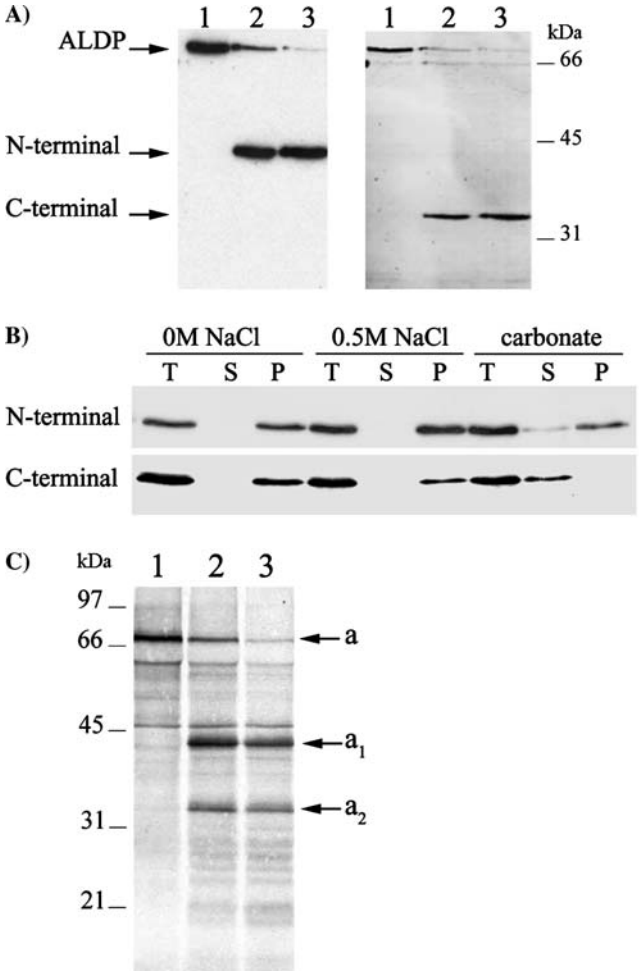

Fig. 1 Characterization of the proteolytic pattern of adrenoleukodystrophy protein (ALDP) after Factor Xa cleavage. **a** Placenta organelles (45 μg of protein) were incubated at 26°C for 45 min in the absence (*lane 1*) or in the presence of Factor Xa at final concentrations of 5 μg/ml and 25 μg/ml (*lanes 2* and *3*, respectively). After trichloroacetic acid (TCA) precipitation, the samples were analyzed by SDS-PAGE followed by Western blotting using two different anti-ALDP antibodies: the monoclonal 1D6 (*left panel*) and a polyclonal directed to the last 18 C-terminal amino acids (*right panel*). This polyclonal antibody presents a weak cross-reactivity with albumin (*faint band* at 66 kDa). The 44-kDa (N-terminal) and the 33-kDa (C-terminal) ALDP fragments are indicated. Molecular mass markers (kDa) are shown on the *right side* of the figure. **b** A placenta organelle fraction, previously digested with Factor Xa, was sonicated in low (0 M NaCl) or high (0.5 M NaCl) ionic buffers or in 0.12 M Na_2CO_3 (carbonate). The samples were halved. One half was kept on ice as a control (*lane T*), and the other half was separated, by centrifugation, into membrane (*lane P*) and soluble fractions (*lane S*). After TCA precipitation, equivalent portions of each fraction (corresponding to 50 μg of the initial protein) were subjected to SDS-PAGE and analyzed as described above. **c** ^{35}S-labelled ALDP was incubated at 26°C for 45 min in the absence (*lane 1*) or in the presence of 5 μg/ml or 25 μg/ml (final concentration) of Factor Xa (*lanes 2, 3*, respectively). The samples were analyzed by SDS-PAGE followed by autoradiography. Full-length ALDP and the two most prominent proteolytic fragments are indicated by *arrows a*, a_1 and a_2, respectively. The positions of the molecular mass standards are indicated on the *left* (kDa)

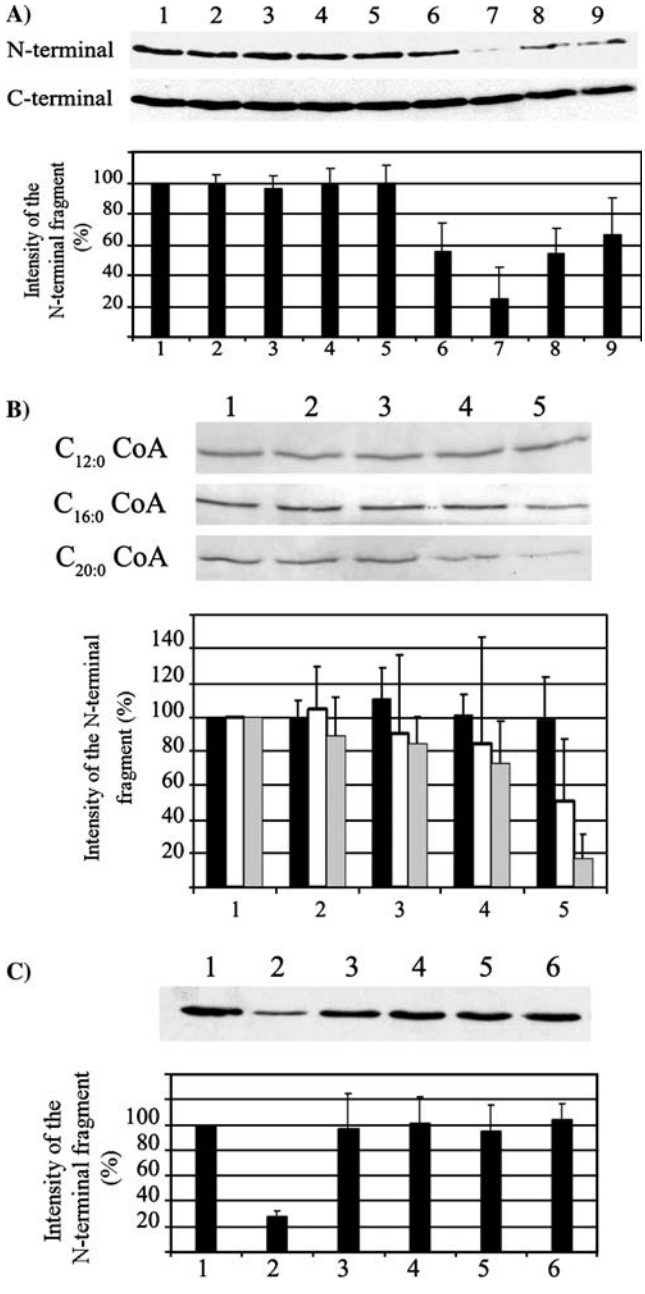

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Fig. 2 Effect of acyl-CoAs esters and free fatty acids on Factor Xa cleavage of adrenoleukodystrophy protein (ALDP). **a** Aliquots of placenta organelles (45 μg of protein) were incubated in cleavage buffer containing 10 mM ATP (*lane 1*) or 10 mM ATP plus 200 μM of one of the following substances: reduced CoA (*lane 2*), acetyl-CoA (*lane 3*), octanoyl-CoA (*lane 4*), dodecanoyl-CoA (*lane 5*), hexadecanoyl-CoA (*lane 6*), eicosanoyl-CoA (*lane 7*), docosanoyl-CoA (*lane 8*), or tetracosanoyl-CoA (*lane 9*). After cleavage with Factor Xa, proteins were analyzed by Western blotting using the monoclonal antibody 1D6 (N-terminal) and the polyclonal antibody directed to the last 18 C-terminal amino acids (C-terminal). A densitometric analysis of the results obtained with the monoclonal antibody 1D6 in three independent experiments is shown (*black bars*). Values were normalized to the control reaction (*lane 1*), which was set to 100%. Standard deviations are also shown. **b** Placenta organelles (45 μg of protein) were incubated in cleavage buffer containing 0, 1, 5, 25, or 200 μM (*lanes 1–5*, respectively) of dodecanoyl-CoA ($C_{12:0}$CoA; *black bars* in the graphic), hexadecanoyl-CoA ($C_{16:0}$CoA; *white bars*) or eicosanoyl-CoA ($C_{20:0}$CoA; *gray bars*). Protein samples were processed as described above using the monoclonal antibody 1D6. **c** Placenta organelles (45 μg of protein) were incubated in cleavage buffer containing 200 μM of the following substances: dodecanoyl-CoA (*lane 1*), eicosanoyl-CoA (*lane 2*), dodecanoic acid (*lane 3*), eicosanoic acid (*lane 4*), dodecanoic acid plus reduced CoA (*lane 5*), and eicosanoic acid plus reduced CoA (*lane 6*). A densitometric analysis of the 44-kDa N-terminal ALDP fragment is shown

milder effects observed with tetracosanoyl-CoA were not expected. In fact, this is one of the fatty acyl groups that accumulate in X-ALD patients (Moser et al. 1999). Thus, if ALDP is involved in the translocation of very-long-chain acyl-CoAs across the peroxisomal membrane (the hypothesis that is being tested here), a larger effect should be observed in the experiment presented in Fig. 2a. It should be noted, however, that several problems were faced when handling docosanoyl-CoA and tetracosanoyl-CoA. In spite of several attempts (see Materials and methods), we were unable to obtain true solutions of these compounds. Thus, the real concentrations of these two acyl-CoAs in the protease assay may be lower than intended. In the experiments described below, only acyl-CoAs presenting the required solubility were used (i.e., CoA thioesters containing acyl groups with 20 or less carbon atoms).

The effects of different concentrations of dodecanoyl-CoA, hexadecanoyl-CoA, and eicosanoyl-CoA on the cleavage of the N-terminal fragment of ALDP by Factor Xa were also assessed. As shown in Fig. 2b, a significant increase in the proteolysis of this ALDP domain is already observed at 25 μM eicosanoyl-CoA. At this concentration, hexadecanoyl-CoA promotes only a minor decrease in the amount of Factor-Xa-resistant ALDP fragment. Dodecanoyl-CoA has no effect at all concentrations tested, as expected.

Finally, when these protease assays were performed in the presence of 200 μM free eicosanoic or dodecanoic fatty acids, no alteration on the sensitivity of the 44-kDa ALDP fragment to the action of Factor Xa was detectable (see Fig. 2c). Thus, the observed phenomenon is directly related to the presence of acyl-CoAs in the protease assay and not to the corresponding free fatty acids, which, in fact, could be generated in our assays by hydrolysis of the added lipids [e.g., from the action of endogenous thioesterases (Hunt and Alexson 2002)].

It should be emphasized that the true concentration of free (unbound) lipids in our assays is not known. In vivo, the total intracellular concentration of acyl-CoAs has been reported to be in the range 5–160 μM (Faergeman and Knudsen 1997). However, under these conditions, most acyl-CoAs are bound to several proteins [e.g., FABP (Faergeman and Knudsen 1997)]. The concentration of these lipid-binding proteins in our assays is surely not sufficient to sequester the exogenously added substrates. Nevertheless, our cleavage buffer does contain the lipid-binding reagent α-cyclodextrin. Thus, the concentration of free lipids in our reactions should also be below their total assay concentration.

ATPγS reverts the acyl-CoA-induced sensitivity of the N-terminal domain of ALDP to proteolysis

The results presented above are compatible with the possibility that CoA-thioesters-possessing acyl groups with 16 or more carbon atoms induce conformational alterations on the N-terminal 44-kDa fragment of ALDP increasing its sensitivity to Factor Xa. There are, however, two other possibilities that must be excluded before drawing such conclusion. The first regards the proteases that are present in these assays. Indeed, the activities of Factor Xa or of some endogenous protease(s) present in the organelle fraction used in these experiments could be stimulated by the acyl-CoAs added to the assays. However, when the activity of Factor Xa was determined in our cleavage assay buffer, in the presence of the acyl-CoAs and/or nucleotides used in this work using a specific substrate (see Materials and methods), no such effect was observed. Furthermore, when Factor Xa is omitted from the assay mixtures, no degradation of ALDP can be observed at 26°C (data not shown). Thus, the proteolytic activities are not variable in our assays. The second possibility that could explain the results described above is related to the physico-chemical properties of the lipids used in this work. Indeed, acyl-CoAs are amphipathic molecules displaying detergent-like properties. Thus, the increased sensitivity of the 44-kDa ALDP fragment to Factor Xa observed in the presence of long- and very-long-chain acyl-CoAs could be due not to conformational alterations of the N-terminal domain of ALDP but rather to physical modifications of its environment. For instance, long- and very-long-chain acyl-CoAs could extract some membrane lipids from the N-terminal domain of ALDP increasing its accessibility to Factor Xa. Data suggesting that this was not the case were obtained when the protease assays were performed in the presence of different nucleotides. As shown in Fig. 3, incubation of ALDP in the presence of ATP or ATPγS, alone or in combination with dodecanoyl-CoA, did not increase the sensitivity of the 44-kDa N-terminal fragment to the action of Factor Xa (compare lane 1 with lanes 2–4, 6). It is also evident that the presence of ATP in the assay mixture does not modify the eicosanoyl-CoA-induced sensitivity of this domain of ALDP to Factor Xa (lane 5). Remarkably, when ATPγS (a poorly hydrolysable ATP analogue) was substituted for ATP, the eicosanoyl-CoA–induced sensitivity of the N-terminal domain of ALDP to Factor Xa was strongly reverted (Fig. 3, compare lanes 5 and 7). Clearly, the presence of ATPγS in the assay mixture blocks the catalytic cycle of ALDP at some step, resulting in a conformation of the 44-kDa N-terminal that is no longer sensitive to Factor Xa. Thus, on one hand, this result indicates the existence of a cross-talk between the C-terminal nucleotide-binding domain of ALDP and its N-terminal domain (the lipid-binding domain); on the other hand, it strongly suggests that acyl-CoAs with 16 or more carbon atoms induce conformational (and not environmental) changes on the 44-kDa N-terminal domain of ALDP.

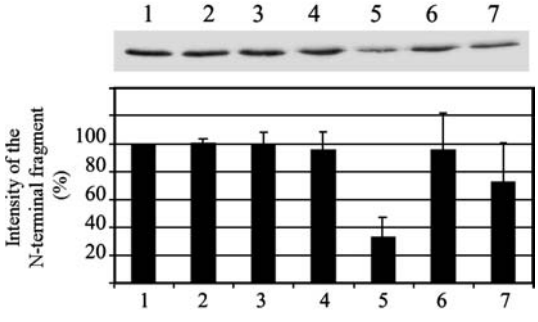

Fig. 3 ATPγS reverts the acyl-CoA-induced sensitivity of the N-terminal domain of adrenoleukodystrophy protein (ALDP) to proteolysis. Human placenta organelles (45 μg of protein) were incubated in cleavage buffer in the absence of lipids and nucleotides (*lane 1*) or in the presence of ATP (*lane 2*), ATPγS (*lane 3*), dodecanoyl-CoA plus ATP (*lane 4*), eicosanoyl-CoA plus ATP (*lane 5*), dodecanoyl-CoA plus ATPγS (*lane 6*), or eicosanoyl-CoA plus ATPγS (*lane 7*). Nucleotides and lipids were used at 10-mM and 200-μM final concentrations, respectively. Protein samples were treated with Factor Xa and subjected to Western blotting using the monoclonal mouse anti-ALDP antibody 1D6. A densitometric analysis from three independent experiments is presented. The means and standard deviations are shown. All the values were normalized to the control reaction (*lane 1*), which was set to 100%

Taken together, our data support the possibility that ALDP plays a direct role in the transport of long- and very-long-chain acyl-CoAs across the peroxisomal membrane.

To conclude, we would like to mention that several attempts were made to obtain additional experimental evidence supporting the observations described above. Specifically, we expressed N-terminal histidine-tagged human ALDP in *Escherichia coli* using the pQE vector (Qiagen). Highly purified ALDP was obtained from inclusion bodies by chromatography using Ni^{2+}-agarose in the presence of 6-M guanidine-HCl, pH 7.0 (data not shown). The aim of these experiments was to reconstitute ALDP in liposomes and to determine its ATPase activity in the presence of different acyl-CoAs and free fatty acids. Unfortunately, all efforts to reconstitute the recombinant protein in phosphatidylcholine/phosphatidylethanolamine vesicles using a dilution method, dialysis, or a solid-phase refolding protocols [reviewed in (Tsumoto et al. 2003)] failed, suggesting that expression of human ALDP in *E. coli* may be a difficult strategy if the aim is to perform functional studies.

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References

Aubourg P, Dubois-Dalcq M (2000) X-linked adrenoleukodystrophy enigma: how does the ALD peroxisomal transporter mutation affect CNS glia? Glia 29:186–190

Bezman L, Moser AB, Raymond GV, Rinaldo P, Watkins PA, Smith KD, Kass NE, Moser HW (2001) Adrenoleukodystrophy: incidence, new mutation rate, and results of extended family screening. Ann Neurol 49:512–517

Braiterman LT, Zheng S, Watkins PA, Geraghty MT, Johnson G, McGuinness MC, Moser AB, Smith KD (1998) Suppression of peroxisomal membrane protein defects by peroxisomal ATP binding cassette (ABC) proteins. Hum Mol Genet 7:239–247

Dean M, Rzhetsky A, Allikmets R (2001) The human ATP-binding cassette (ABC) transporter superfamily. Genome Res 11:1156–1166

Dubois-Dalcq M, Feigenbaum V, Aubourg P (1999) The neurobiology of X-linked adrenoleukodystrophy, a demyelinating peroxisomal disorder. Trends Neurosci 22:4–12

Faergeman NJ, Knudsen J (1997) Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. Biochem J 323:1–12

Footitt S, Slocombe SP, Larner V, Kurup S, Wu Y, Larson T, Graham I, Baker A, Holdsworth M (2002) Control of germination and lipid mobilization by COMATOSE, the Arabidopsis homologue of human ALDP. Embo J 21:2912–2922

Fujiki Y, Hubbard AL, Fowler S, Lazarow PB (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J Cell Biol 93:97–102

Gouveia AM, Reguenga C, Oliveira ME, Sa-Miranda C, Azevedo JE (2000) Characterization of peroxisomal Pex5p from rat liver. Pex5p in the Pex5p–Pex14p membrane complex is a transmembrane protein. J Biol Chem 275:32444–32451

Guimaraes CP, Lemos M, Menezes I, Coelho T, Sa-Miranda C, Azevedo JE (2001) Characterisation of two mutations in the ABCD1 gene leading to low levels of normal ALDP. Hum Genet 109:616–622

Guimaraes CP, Domingues P, Aubourg P, Fouquet F, Pujol A, Jimenez-Sanchez G, Sa-Miranda C, Azevedo JE (2004) Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo. Biochim Biophys Acta 1689:235–243

Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc Natl Acad Sci USA 97:7754–7759

Hettema EH, van Roermund CW, Distel B, van den Berg M, Vilela C, Rodrigues-Pousada C, Wanders RJ, Tabak HF (1996) The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of Saccharomyces cerevisiae. Embo J 15:3813–3822

Hunt MC, Alexson SE (2002) The role Acyl-CoA thioesterases play in mediating intracellular lipid metabolism. Prog Lipid Res 41:99–130

Jones PM, George AM (2004) The ABC transporter structure and mechanism: perspectives on recent research. Cell Mol Life Sci 61:682–699

Julien M, Gros P (2000) Nucleotide-induced conformational changes in P-glycoprotein and in nucleotide binding site mutants monitored by trypsin sensitivity. Biochemistry 39:4559–4568

Kemp S, Pujol A, Waterham HR, van Geel BM, Boehm CD, Raymond GV, Cutting GR, Wanders RJ, Moser HW (2001) ABCD1 mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. Hum Mutat 18:499–515

Lazarow PB, De Duve C (1976) A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. Proc Natl Acad Sci USA 73:2043–2046

Manciu L, Chang XB, Buyse F, Hou YX, Gustot A, Riordan JR, Ruysschaert JM (2003) Intermediate structural states involved in MRP1-mediated drug transport. Role of glutathione. J Biol Chem 278:3347–3356

McGuinness MC, Lu JF, Zhang HP, Dong GX, Heinzer AK, Watkins PA, Powers J, Smith KD (2003) Role of ALDP (ABCD1) and mitochondria in X-linked adrenoleukodystrophy. Mol Cell Biol 23:744–753

Moser AB, Kreiter N, Bezman L, Lu S, Raymond GV, Naidu S, Moser HW (1999) Plasma very long chain fatty acids in 3,000 peroxisome disease patients and 29,000 controls. Ann Neurol 45:100–110

Moser HW, Smith KD, Watkins PA, Powers J, Moser AB (2001) X-linked adrenoleukodystrophy. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 3257–3302

Mosser J, Douar AM, Sarde CO, Kioschis P, Feil R, Moser H, Poustka AM, Mandel JL, Aubourg P (1993) Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature 361:726–730

Mosser J, Lutz Y, Stoeckel ME, Sarde CO, Kretz C, Douar AM, Lopez J, Aubourg P, Mandel JL (1994) The gene responsible for adrenoleukodystrophy encodes a peroxisomal membrane protein. Hum Mol Genet 3:265–271

Shani N, Valle D (1996) A Saccharomyces cerevisiae homolog of the human adrenoleukodystrophy transporter is a heterodimer of two half ATP-binding cassette transporters. Proc Natl Acad Sci USA 93:11901–11906

Shani N, Watkins PA, Valle D (1995) PXA1, a possible Saccharomyces cerevisiae ortholog of the human adrenoleukodystrophy gene. Proc Natl Acad Sci USA 92:6012–6016

Tsumoto K, Ejima D, Kumagai I, Arakawa T (2003) Practical considerations in refolding proteins from inclusion bodies. Protein Expr Purif 28:1–8

Verleur N, Hettema EH, van Roermund CW, Tabak HF, Wanders RJ (1997) Transport of activated fatty acids by the peroxisomal ATP-binding-cassette transporter Pxa2 in a semi-intact yeast cell system. Eur J Biochem 249:657–661

Wanders RJ (2004) Metabolic and molecular basis of peroxisomal disorders: a review. Am J Med Genet 126A:355–375

Wang G, Pincheira R, Zhang JT (1998) Dissection of drug-binding-induced conformational changes in P-glycoprotein. Eur J Biochem 255:383–390

Watkins PA, Gould SJ, Smith MA, Braiterman LT, Wei HM, Kok F, Moser AB, Moser HW, Smith KD (1995) Altered expression of ALDP in X-linked adrenoleukodystrophy. Am J Hum Genet 57:292–301

Zolman BK, Silva ID, Bartel B (2001) The Arabidopsis pxa1 mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid beta-oxidation. Plant Physiol 127:1266–1278