The peroxisomal protein import machinery displays a preference for monomeric substrates

Marta O. Freitas¹,†, Tânia Francisco¹,†, Tony A. Rodrigues¹,², Celien Lismont⁴, Pedro Domingues³, Manuel P. Pinto¹, Cláudia P. Grou¹, Marc Fransen⁴ and Jorge E. Azevedo¹,²

¹Organelle Biogenesis and Function Group, Instituto de Biologia Celular e Molecular (IBMC), Universidade do Porto, Porto, Portugal
²Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal
³Departamento de Química, Universidade de Aveiro, Aveiro, Portugal
⁴Departement Cellulaire en Moleculaire Geneeskunde, KU Leuven – Universiteit Leuven, Leuven, Belgium

1. Summary

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and transported by the shuttling receptor PEX5 to the peroxisomal membrane docking/translocation machinery, where they are translocated into the organelle matrix. Under certain experimental conditions this protein import machinery has the remarkable capacity to accept already oligomerized proteins, a property that has heavily influenced current models on the mechanism of peroxisomal protein import. However, whether or not oligomeric proteins are really the best and most frequent clients of this machinery remain unclear. In this work, we present three lines of evidence suggesting that the peroxisomal import machinery displays a preference for monomeric proteins. First, in agreement with previous findings on catalase, we show that PEX5 binds newly synthesized (monomeric) acyl-CoA oxidase 1 (ACOX1) and urate oxidase (UOX), potently inhibiting their oligomerization. Second, in vitro import experiments suggest that monomeric ACOX1 and UOX are better peroxisomal import substrates than the corresponding oligomeric forms. Finally, we provide data strongly suggesting that although ACOX1 lacking a peroxisomal targeting signal can be imported into peroxisomes when co-expressed with ACOX1 containing its targeting signal, this import pathway is inefficient.

2. Introduction

Peroxisomes are single membrane-bounded organelles that participate in many biochemical pathways [1–4]. In mammals, they have a relatively simple protein repertoire, harbouring about 50 enzymes in their matrix [5,6]. All these proteins are synthesized on cytosolic ribosomes and post-translationally targeted to the organelle matrix [7]. Their correct sorting relies on one of two peroxisomal targeting signals (PTS): the PTS type 1 (PTS1), a C-terminal peptide generally ending with the sequence Ser-Lys-Leu, found in the vast majority of peroxisomal matrix proteins [8,9]; and the PTS2, a degenerate nonapeptide present at the N-termini of just a few proteins [10–12].

According to current models [13–16], newly synthesized peroxisomal matrix proteins are recognized by the shuttling receptor PEX5 while still in the cytosol [17]. PTS1 proteins bind directly to PEX5, whereas PTS2 proteins require the ancillary protein PEX7 to interact with PEX5 [8,12,18–21]. Following this recognition event, the PEX5/PEX7–cargo protein complex interacts with the docking/translocation machinery (DTM) [22–24], a multisubunit transmembrane protein complex of the
oligomers in order to be accepted by the PIM [55]. Finally, some studies clearly showing that cargo proteins do not have to contain a PTS is also imported into peroxisomes. These findings it was subsequently reported that (monomeric) tetrameric enzyme have been provided [54]. In line with these findings, the protein import machineries of mitochondria and endoplasmic reticulum, has the capacity to accept bulky/already folded clients [39,40]. However, in the absence of additional data, it remains unclear how frequent and efficient the import of mal proteins are imported as oligomers is also fed by a premature oligomerization of those proteins in the cytosol prior to import, then import of oligomeric cargoes becomes the rule for protein translocation across the peroxisomal membrane, because most peroxisomal matrix proteins are indeed homo-oligomers [48]. This is the scenario behind some previous models proposing that cargoes (containing multiple PTSs due to their oligomeric nature) are presented to the DTM by multiple molecules of PEX5 [48,49]. If, instead, we assume that under normal physiological conditions newly synthesized matrix proteins are kept in a monomeric near-native conformation until they arrive at the organelle matrix, then a model in which a single PEX5 molecule delivers a single cargo to the DTM is more likely [59,60]. The outcomes of each of these assumptions to the cargo protein translocation step are quite different because, as stated above, all the available data suggest that cargo proteins are translocated across the organelle membrane by PEX5 itself, when the receptor becomes inserted into the DTM. Thus, the first scenario would predict that each oligomeric cargo is translocated by several PEX5 molecules (see [15] for a mechanism of this type), whereas in the second scenario a single PEX5 molecule would suffice [59].

Previously, we found that PEX5 at physiological concentrations binds monomeric catalase, potently blocking its tetramerization [61]. This property, together with the fact that there is sufficient PEX5 in rat hepatocyte cytosol to bind all newly synthesized peroxisomal matrix proteins, led us to hypothesize that PEX5, in addition to its role as a receptor and translocator, is also a chaperone/holdase, binding newly synthesized monomeric proteins in the cytosol and inhibiting premature or incorrect interactions [61]. In this work, we have characterized the import pathway of acyl-CoA oxidase 1 (ACOX1; a homo-dimeric protein in its native state [62]) and urate oxidase (UOX; a homo-tetramer [63]), two peroxisomal matrix proteins which together with catalase comprise one-third of the total protein molecules found in mouse/rat liver peroxisomal matrix [62,64]. We found that PEX5 also binds the monomeric version of these proteins, blocking their homo-oligomerization. Importantly, peroxisomal import assays suggest that the monomeric versions of ACOX1 and UOX are much better substrates for the PIM than the corresponding homo-oligomeric versions. Altogether, these results suggest that import of monomeric proteins into the peroxisome is not a phenomenon restricted to a few particular clients. Rather, at the very least, our data raise the possibility that many of the protein translocation events occurring at the PIM involve monomeric cargoes.

3. Results

3.1. PEX5 inhibits dimerization of newly synthesized acyl-CoA oxidase 1

We have recently shown that a rabbit reticulocyte lysate-based in vitro translation system can be used to prepare monomeric and tetrameric versions of catalase. The amount there are at least three peroxisomal matrix proteins that no longer bind PEX5 upon oligomerization. These are alcohol oxidase from Hansenula polymorpha and mammalian carbonyl reductase and epoxide hydrolase [56–58]. Seemingly, at least in these cases, the proteins have to remain monomeric in order to be imported into peroxisomes.

Determining the type of substrate preferred by the PIM is of major importance to understand its mechanism. If we assume that almost all oligomeric peroxisomal proteins oligomerize in the cytosol prior to import, then import of oligomeric cargoes becomes the rule for protein translocation across the peroxisomal membrane, because most peroxisomal matrix proteins are indeed homo-oligomers [48]. This is the scenario behind some previous models proposing that cargoes (containing multiple PTSs due to their oligomeric nature) are presented to the DTM by multiple molecules of PEX5 [48,49]. If, instead, we assume that under normal physiological conditions newly synthesized matrix proteins are kept in a monomeric near-native conformation until they arrive at the organelle matrix, then a model in which a single PEX5 molecule delivers a single cargo to the DTM is more likely [59,60]. The outcomes of each of these assumptions to the cargo protein translocation step are quite different because, as stated above, all the available data suggest that cargo proteins are translocated across the organelle membrane by PEX5 itself, when the receptor becomes inserted into the DTM. Thus, the first scenario would predict that each oligomeric cargo is translocated by several PEX5 molecules (see [15] for a mechanism of this type), whereas in the second scenario a single PEX5 molecule would suffice [59].

Previously, we found that PEX5 at physiological concentrations binds monomeric catalase, potently blocking its tetramerization [61]. This property, together with the fact that there is sufficient PEX5 in rat hepatocyte cytosol to bind all newly synthesized peroxisomal matrix proteins, led us to hypothesize that PEX5, in addition to its role as a receptor and translocator, is also a chaperone/holdase, binding newly synthesized monomeric proteins in the cytosol and inhibiting premature or incorrect interactions [61]. In this work, we have characterized the import pathway of acyl-CoA oxidase 1 (ACOX1; a homo-dimeric protein in its native state [62]) and urate oxidase (UOX; a homo-tetramer [63]), two peroxisomal matrix proteins which together with catalase comprise one-third of the total protein molecules found in mouse/rat liver peroxisomal matrix [62,64]. We found that PEX5 also binds the monomeric version of these proteins, blocking their homo-oligomerization. Importantly, peroxisomal import assays suggest that the monomeric versions of ACOX1 and UOX are much better substrates for the PIM than the corresponding homo-oligomeric versions. Altogether, these results suggest that import of monomeric proteins into the peroxisome is not a phenomenon restricted to a few particular clients. Rather, at the very least, our data raise the possibility that many of the protein translocation events occurring at the PIM involve monomeric cargoes.

3. Results

3.1. PEX5 inhibits dimerization of newly synthesized acyl-CoA oxidase 1

We have recently shown that a rabbit reticulocyte lysate-based in vitro translation system can be used to prepare monomeric and tetrameric versions of catalase. The amount
of each of these species in translation reactions is time-dependent: synthesis reactions performed for a short period of time yielded essentially monomeric catalase; longer incubations led to the conversion of a fraction of the monomeric protein into tetrameric catalase, a process that was strongly inhibited by PEX5 [61]. Here, we determined whether the same experimental strategy could be applied to other oligomeric peroxisomal matrix proteins. The aim was twofold: (i) to characterize the effect of PEX5 on their oligomerization process and (ii) to obtain monomeric and oligomeric versions of these proteins so that their in vitro peroxisomal import competences could be compared (note that all our attempts to import monomeric or tetrameric catalase into rat/mouse liver peroxisomes have failed thus far, probably because the PEX5–catalase interaction is too transient, and therefore too sensitive to the competition exerted by endogenous (liver) soluble PTS1 proteins present in these in vitro assays; T Francisco, JE Azevedo, unpublished observations, see also [23]). We focused mainly on ACOX1, but some experiments were repeated with another peroxisomal matrix protein (see later). Native ACOX1 comprises two identical 74 kDa subunits, each of which is partially and slowly cleaved in the peroxisomal matrix in vivo into an N-terminal domain of 53 kDa and a C-terminal 21 kDa polypeptide [65–67].

We first used an immunoprecipitation assay to assess whether ACOX1 can interact with itself in the in vitro translation system. As shown in figure 1a, upper panel, when ACOX1 and an epitope-tagged version of it containing two haemagglutinin (HA) sequences at the N-terminus (2HA-ACOX1) were co-synthesized in vitro for 30 min, chased in the presence of cycloheximide for 4 h, and subject to immunoprecipitation using an anti-HA antibody, a significant amount of ACOX1 was co-immunoprecipitated with 2HA-ACOX1 (lane 7). No ACOX1 was recovered in immunoprecipitates when the two proteins were synthesized separately for 30 min and mixed just before immunoprecipitation, or when the co-synthesis and chase incubations were performed in the presence of recombinant PEX5 (figure 1a, upper panel, lanes 6 and 8, respectively). For reasons that will become apparent below, we used the same strategy to determine whether 2HA-ACOX1 can interact with an ACOX1 species possessing a Flag epitope at its C-terminus (ACOX1-Flag). The results shown in figure 1a, lower panel, suggest that this is indeed the case.

In another approach, we used sucrose gradient centrifugation to characterize the sedimentation behaviour of in vitro synthesized ACOX1. As shown in figure 1b, ACOX1 synthesized for just 30 min sedimented as a globular monomeric protein, together with albumin, a 67 kDa protein (panel I, lane 6). We refer to this species as monomeric ACOX1 (mACOX1). When mACOX1 was chased for 4 h in the presence of cycloheximide, a second ACOX1 population was detected in these gradients (figure 1b, panel II, lane 9). Its sedimentation behaviour is identical to the one of native/dimeric mouse liver ACOX1 (figure 1b, panel V, bands marked ACOX1a/b/c in lane 9). This species is hereafter referred to as dimeric ACOX1 (dACOX1). Centrifugation of the two 35S-labelled ACOX1 populations in the presence of recombinant PEX5 resulted in a slight increase in their sedimentation coefficients (figure 1b, panel III), suggesting that both species interact with PEX5. In agreement with the data shown in figure 1a, less dACOX1 was generated in translation reactions chased for 4 h in the presence of PEX5. In this case, the major ACOX1 population was detected in fractions 7–8, a behaviour identical to the one observed for mACOX1 in the sucrose gradient containing PEX5 (figure 1b, compare panels III and IV).

To further characterize the two ACOX1 populations detected in these experiments their resistance to proteolysis was assessed. As shown in figure 1c (left panel) 35S-mACOX1 is quite sensitive to proteinase K. By contrast, 35S-dACOX1 is cleaved by the protease into a major 51 kDa fragment plus three fragments of 40, 32 and 21 kDa (figure 1c, right panel). Importantly, an almost identical proteolysis pattern was obtained for native mouse liver dimeric ACOX1, as assessed by western blotting using antibodies directed to the 53 kDa and 21 kDa polypeptides (figure 1d). The only difference between the 35S-dACOX1 species and endogenous mouse dACOX1 after protease treatment resides in the relative intensity of the 21 kDa fragment which is barely detectable in the 35S-labelled protein. The fact that this domain of ACOX1 contains only one of the 19 methionines present in full-length ACOX1 justifies this difference.

In summary, the experiments described above suggest the following: (i) ACOX1 synthesized in vitro for a short period of time is a globular monomeric protease-sensitive protein; (ii) further incubation of in vitro synthesized mACOX1 results in its partial conversion into dACOX1; and (iii) PEX5 inhibits ACOX1 dimerization.

3.2. Peroxisomal in vitro import efficiencies of mACOX1 and dACOX1

We next tested the peroxisomal import efficiencies of 35S-mACOX1 and 35S-dACOX1 using an established in vitro import system. For this purpose mACOX1 and dACOX1 obtained from a sucrose gradient as the one presented in figure 1b (panel II) were incubated with a rat liver post-nuclear supernatant (PNS) in import buffer supplemented with 1.5 nM recombinant PEX5 (see Material and methods for details). Two control import reactions were included in these experiments. In the first, the import buffer contained also 300 nM of a recombinant protein comprising the C-terminal half of PEX5 (hereafter referred to as TPRs). This truncated PEX5 protein retains the capacity to interact with PTS1-containing proteins but lacks peroxisomal targeting information [68]. Thus, if the concentration of TPRs is much larger than the concentration of PEX5 in the assays, then import of 35S-labelled ACOX1 should be strongly inhibited. The second control reaction received 300 nM of an inactive version of TPRs (TPRs(N526K)), a protein containing a single missense mutation (N526K) that abolishes its PTS1-binding capacity [18,69]. This recombinant protein should not inhibit PEX5-dependent import of ACOX1. At the end of the incubation, import reactions were treated or not with a large amount of proteinase K and the organelles were isolated by centrifugation and analysed by SDS-PAGE/autoradiography. The results of this experiment are shown in figure 2a. Approximately 50% of mACOX1 sedimenting with the organelles acquired a protease-resistant status (compare lanes 1 and 7). An identical result was obtained in the reaction supplemented with TPRs(N526K) (compare lanes 3 and 9), as expected. By contrast, the amount of protease-protected mACOX1 in the reaction supplemented with TPRs was strongly diminished (compare lanes 7 and 8). A different result was obtained for dACOX1. Indeed, although a significant fraction of this protein sedimented with the organelles (lanes 4–6), the vast majority of it remained accessible to the protease (lanes 10–12), indicating that it was not
translocated across a membrane. Note that a very small amount of intact dACOX1 is detected in these samples. However, this material is unresponsive to recombinant TPRs (compare lane 11 with lanes 10 and 12) and, therefore, it does not represent authentic imported protein. In agreement with this interpretation, the amount of uncleaved dACOX1 in import assays...
a sucrose gradient and native ACOX1 (from mouse liver purified peroxisomes) were subjected to protease treatment in the presence of Triton X-100 and subjected to transcription/translation. (b) Sedimentation behaviour of in vitro synthesized ACOX1. ACOX1 synthesized for 30 min (panel I), and ACOX1 synthesized for 30 min and chased for 4 h in the absence (panels II and III) or presence of 1 mM PEX5 (panel IV) were loaded onto the top of sucrose gradients supplemented with 1 mM of either PEX5 (panels III and IV) or a control protein (panels I and II). After centrifugation, fractions were collected from the bottom of the gradients and subjected to SDS-PAGE/autoradiography. Ovalbumin (OA), bovine serum albumin (BSA) and immunoglobulins (IgGs) were used as sedimentation coefficient standards. Peroxosomal matrix proteins from mouse liver were also subjected to this analysis. A Coomassie-stained gel is shown (panel V). Carbamoyl phosphate synthetase (CPS), 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (EHHADH), acyl-CoA oxidase I subunits a, b and c (ACOX1a, ACOX1b, ACOX1c, respectively) and catalase (Cat) were identified by nano-HPLC-MALDI-MS/MS (data not shown). (c) Monomeric and dimeric ACOX1 present different susceptibilities to protease K. 35S-mACOX1 and 35S-dACOX1 isolated from a sucrose gradient were treated with increasing concentrations of protease K (PK) for 40 min on ice. After protease inactivation, samples were analysed by SDS-PAGE/autoradiography. Numbers to the left indicate the molecular weights of protein standards. Arrow heads indicate proteolysis fragments of ACOX1 (see main text). (d) Dimeric 35S-ACOX1 and native/peroxisomal ACOX1 display the same proteolysis profile. 35S-dACOX1 isolated from a sucrose gradient and native ACOX1 (from mouse liver purified peroxisomes) were subjected to protease treatment in the presence of Triton X-100 and subjected to SDS-PAGE/autoradiography (left panel) or western blotting using antibodies directed to the 53-kDa ACOX1 polypeptide (central panel). The same blot was reprobed with an antibody directed to the 21-kDa polypeptide of ACOX1 (right panel). F, front of the gel.

Figure 2. (a) 35S-mACOX1 is a better peroxisomal import substrate than dACOX1. (a) 35S-mACOX1 and 35S-dACOX1 isolated from a sucrose gradient were subjected to in vitro import reactions in the presence (+) or absence (−) of the indicated recombinant proteins. After incubation, one-half of each sample was treated with proteinase K, as indicated. The organelles were then isolated and analysed by SDS-PAGE/autoradiography. The autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. 5, 6—5% of 35S-mACOX1 and 35S-dACOX1, respectively, used in the assays. The arrow head indicates the 51 kDa protease-resistant fragment of 35S-dACOX1. (b) Import kinetic analyses of 35S-mACOX1 and 35S-dACOX1. The two import reactions (each containing 2.5 mg of PNS) were performed in the presence of recombinant PEX5. Aliquots of each import reaction (containing 500 μg of PNS) were withdrawn at the indicated time points, treated with proteinase K and analysed as above. Note that the amount of 35S-dACOX1 used in this experiment was approximately twofold that of 35S-mACOX1 to obtain similar substrate concentrations. Lane 1—5% of the radiolabelled proteins present in each aliquot does not increase over time, in contrast to mACOX1 import (figure 2b).

Taken together, the results of these in vitro import experiments, although of qualitative nature, strongly indicate that mACOX1 is a far better substrate for the PIM than dACOX1.

3.3. Urate oxidase behaves similarly to ACOX1 in the in vitro homo-oligomerization and import assays

Aiming at extending the findings obtained with ACOX1 to another peroxisomal protein, we tested UOX in some of the assays described above. UOX, an abundant protein comprising 15% of the total protein molecules found in rat/mouse liver peroxisomes, is a homo-tetramer of 35 kDa subunits in its native state [63,64]. We first asked whether UOX can homooligomerize in the rabbit reticulocyte lysate and, if so, whether this process is inhibited by PEX5. The strategy used was exactly the one described above for ACOX1 (figure 1a). As shown in figure 3a, untreated UOX was co-immunoprecipitated with 2HA-UOX only when the two proteins were co-synthesized in the absence of PEX5 (lane 7). Thus, PEX5 blocks UOX oligomerization.

35S-UOX synthesized for just 45 min and 35S-UOX subjected to a 4-h chase incubation were also analysed by sucrose gradient centrifugation. As shown in figure 3b, the first protein (hereafter referred to as monomeric UOX; mUOX) sedimented slightly above ovalbumin, a 45 kDa monomeric globular protein (panel I, lanes 4 and 5), whereas a fraction of the protein that was allowed to oligomerize (referred to as tetrameric UOX; tUOX) sedimented as authentic native/tetrameric mouse liver UOX (panel II, lane 9; compare with panel V in figure 1b). The two species of UOX display quite different behaviours upon proteinase K treatment: mUOX is readily degraded by the protease, whereas tUOX is largely resistant yielding a diffuse doublet that runs slightly below undigested UOX upon SDS-PAGE (figure 3c, left and right panels, respectively).

Finally, 35S-mUOX and 35S-UOX obtained from a sucrose gradient were tested in in vitro import assays. The criteria to
define *bona fide* peroxisomal import were the ones used above for ACOX1, i.e. acquisition of a protease-protected, organelle-associated status in a TPRs-inhibitable manner. As shown in figure 3d, a small amount of mUOX was imported into peroxisomes (cf. lanes 4 and 6 with 5). By contrast, we were unable to detect specific peroxisomal import of tUOX. Indeed, the radio-labelled protein appearing in the organelle pellets is insensitive to the presence of TPRs in the assays (cf. lanes 10 and 11) and runs as a diffuse doublet upon SDS-PAGE, indicating that it is protease-accessible.

**Figure 3.** Behaviour of UOX in *in vitro* homo-oligomerization and import assays. (a) *In vitro* synthesized UOX oligomerizes in a process inhibited by PEX5. UOX and HA-tagged UOX (2HA-UOX) were synthesized individually (lanes 1 and 2) or co-synthesized in the absence or presence of 1 μM recombinant PEX5 (lanes 4 and 5, respectively) for 45 min, and subjected to a 4 h chase. A mixture of the two proteins synthesized individually (lane 3) and the co-synthesis reactions (lanes 4 and 5) were subjected to immunoprecipitation (IP) using anti-HA antibody agarose beads (lanes 6–8, respectively) and analysed as described in figure 1a. IVT, *in vitro* transcription/translation. (b) Sedimentation analyses of *in vitro* synthesized UOX. Radiolabelled UOX synthesized for 45 min (panel I) or UOX synthesized for 45 min and chased for 4 h (panel II) were subjected to sucrose gradient centrifugation analyses. The sedimentation positions of ovalbumin (OA), bovine serum albumin (BSA) and immunoglobulins (IgGs) are also shown. (c) Monomeric and tetrameric UOX display different susceptibilities to protease treatment. $^{35}$S-mUOX and $^{35}$S-tUOX isolated from a sucrose gradient were subjected to proteinase K (PK) treatment (400 μg ml$^{-1}$, final concentration) and aliquots were withdrawn at the indicated time points. Samples were processed as described in figure 1c. (d) mUOX is a better substrate for the peroxisomal protein import machinery than tUOX. $^{35}$S-mUOX and $^{35}$S-tUOX isolated from a sucrose gradient were subjected to *in vitro* import assays in the presence (+) or absence (−) of the indicated recombinant proteins. After incubation, one-half of each sample was treated with proteinase K, as indicated. Isolated organelles were processed and analysed by SDS-PAGE/autoradiography. The autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. I1, I2—5% of $^{35}$S-mUOX and $^{35}$S-tUOX, respectively, used in the assays. The arrow heads indicate protease-resistant fragments of $^{35}$S-tUOX.
3.4. ACOX1 lacking peroxisomal targeting information can be imported into peroxisomes piggybacked with ACOX1 containing the PTS1, but this pathway is inefficient

The in vitro import assays described above suggest that mACOX1 is a much better substrate for the PIM than dACOX1. An important question is whether this preference is maintained under in vivo conditions. To address this issue, we (co-)transfected COS-7 cells with plasmids encoding two epitope-tagged versions of ACOX1, one containing a functional PTS1 (2HA-ACOX1; see above) and the other lacking it (ACOX1-Flag). Next, we investigated whether the first protein can carry the second one to the peroxisome, and if so, with what efficiency. Control experiments (figure 4a), in which...
each of these plasmids was transfected alone, revealed a peroxi-
osomal (panel I) and cytosolic (panel II) staining pattern for
2HA-ACOX1 and ACOX1-Flag, respectively. Interestingly, in
a small number of cells (less than 5%), expression of ACOX1-
Flag also resulted in a staining pattern that partially, but very
weakly, overlapped with that of the peroxisomal marker
protein PEX14 (figure 4a, panel III), suggesting that a minor
amount of ACOX1-Flag was imported into peroxisomes piggy-
backed with endogenous ACOX1 (see below). In agreement
with the peroxisomal localization observed for 2HA-ACOX1,
western blot analyses of total cell extracts using an antibody
directed to the 53 kDa polypeptide of ACOX1 revealed that
the majority of this protein ran below the intact 74 kDa
endothelial ACOX1 and immediately above its 53 kDa frag-
ment, indicating that it was cleaved in the peroxisomal matrix
(figure 4b). We next co-transfected cells with mixtures of
the two plasmids, and determined the subcellular localization of
each ACOX1 species by immunofluorescence at 1, 2 and 3
days post-transfection. To ensure that most ACOX1-Flag
produced in these cells had the possibility to interact with
newly synthesized 2HA-ACOX1, and thus to be imported
into peroxisomes, a 1 : 10 mixture of the expression plasmids
encoding ACOX1-Flag and 2HA-ACOX1, respectively, was
used. Under these conditions, an exclusive peroxisomal locali-
zation was found for 2HA-ACOX1 regardless of the time point
at which the immunofluorescence analyses were performed.
By contrast, ACOX1-Flag displayed a cytosolic localization in
more than 90% of the cells analysed at 1 day post-transfection
(figure 4c, bar graph at the left-hand side). Interestingly, how-
ever, a small percentage of cells displayed a dual cytosolic and
peroxisomal localization for ACOX1-Flag at this time point.
The fraction of cells displaying such a distribution pattern
increased over the next 2 days, but an exclusive peroxisomal localization for ACOX1-Flag could never be observed.

We were able to increase significantly the percentage of
cells presenting a peroxisomal localization for ACOX1-
Flag by transfecting cells with a 1 : 30 mixture of the plasmids
encoding ACOX1-Flag and 2HA-ACOX1, respectively. As
shown in figure 4c (bar graph at the right-hand side), an ex-
sclusive peroxisomal localization for ACOX1-Flag was found in
approximately 5% of cells already at 1 day post-transfection.
Similarly to the results above, the fraction of cells presenting this
labelling pattern increased slowly during the two subsequent
days. Together, these experiments strongly indicate that
ACOX1-Flag can interact with 2HA-ACOX1 in the cytosol
and use its PTS1 to reach the peroxisome. Thus, dimeric
ACOX1 is also a substrate for the PIM. However, it is also
evident from these data that targeting of ACOX1-Flag to the
peroxisome, in contrast to that of 2HA-ACOX1, is a low-efficiency process occurring over a timescale of days.

The reason why ACOX1-Flag is poorly imported into the
gonad could reflect difficulties of the Flag-tagged protein in
interacting with 2HA-ACOX1. Although the in vitro oligo-
erization assay shown in figure 1a already suggests that this is not the case, an in vitro approach was used to test this
possibility. Specifically, we co-expressed ACOX1-Flag with a
2HA-ACOX1 species containing three copies of a nuclear-
localization signal (NLS) at its C-terminus (thus blocking its
PTS1; see figure 4a, panel IV) in COS-7 cells and asked whether
the nuclear targeted protein (2HA-ACOX1–3NLS) could trans-
port ACOX1-Flag to the nucleus. Similarly to the experiment
above, we used a 1 : 10 mixture of the plasmids encoding
ACOX1-Flag and 2HA-ACOX1–3NLS, respectively. As
shown in figure 4d, almost all ACOX1-Flag was found in the
nucleus, thus suggesting that the Flag epitope does not interfere with the dimerization of ACOX1.

4. Discussion

The idea that most newly synthesized peroxisomal proteins are
imported into the gonad after oligomerization in the cytosol
has remained widely accepted during the last two decades.
Besides all the studies referred to above that were used to
develop and support this concept (see Introduction section),
other arguments were frequently used to strengthen it. An
important one, which is nowadays questionable [70,71], was
that peroxisomes seemed to lack a protein-folding machinery
[45,48,55]. Thus, newly synthesized peroxisomal matrix pro-
teins should undergo folding and oligomerization in the
cytoplasm, where such a machinery exists. Data on human alanine-glyoxylate amino transferase, a peroxisomal homo-
dimeric enzyme, seemed to provide the proof-of-concept for
this idea. Indeed, some mutations in the enzyme found in
hyperoxaraluria type-I patients lead to the mistargeting of a frac-
tion of the protein to the mitochondria. Since these mutations
also affect dimerization of the enzyme, it was thus concluded
that only the dimeric enzyme is competent for peroxiso-
mal import (reviewed in [72,73]). However, an identical
mistargeting phenomenon was recently described for human

---

Figure 4. (Overleaf) ACOX1 lacking a peroxisomal targeting signal is inefficiently imported into peroxisomes when co-expressed with ACOX1 containing a PTS1. (a) COS-7 cells were transfected with plasmids encoding HA-tagged ACOX1 (2HA-ACOX1; panel I), a C-terminally Flag-tagged ACOX1 (ACOX1-Flag; panels II and III), or a HA-tagged ACOX1 containing a nuclear targeting sequence (2HA-ACOX1–3NLS; panel IV). Two days post-transfection the cells were fixed, counterstained with
DAPI, and processed for immunofluorescence using an anti-PEX14 antibody (to label peroxisomes) and an anti-HA (panels I and IV) or anti-Flag antibody (panels II and III). Profile plots of fluorescence intensity (in percentage of pixel intensity) along the white arrows shown in the merged panels are also provided: blue line, DAPI staining; green line, anti-PEX14 fluorescence; red line, anti-HA or anti-Flag fluorescence. Scale bar, 10 µm. (b) Expression levels of tagged ACOX1 proteins in
COS-7 cells. Untransfected cells (lanes ‘−−’) or cells transfected with individual plasmids encoding ACOX1-Flag, 2HA-ACOX1 or 2HA-ACOX1–3NLS were analysed by
western blot using an antibody against the 53 kDa polypeptide of ACOX1. The arrow heads indicate the tagged ACOX1 proteins. (c) COS-7 cells were transfected with
mixtures of the plasmids encoding ACOX1-Flag and 2HA-ACOX1 at ratios of 1 : 10 and 1 : 30. Note that, as the total amount of plasmid in each of these mixtures was
adjusted to 1 µg, ‘1’ corresponds to 10% and 3.3%, respectively, of the amount of plasmid DNA used in (a) and (b). The subcellular localization of each protein was
then analysed by immunofluorescence 1, 2 and 3 days post-transfection (dpt). Cells in which ACOX1-Flag displays an exclusive cytosolic localization (Cyt; see upper
panel for a representative example), a dual peroxisomal/cytosolic localization (PO/Cyt; middle panel), or an exclusive peroxisomal localization (PO; lower panel) were
counted and expressed as percentage of ACOX1-Flag-expressing cells in the bar graph. (d) Exactly the same co-transfection strategy was used with plasmids encod-
ing 2HA-ACOX1–3NLS and ACOX1-Flag. Cells in which ACOX1-Flag displays an exclusive cytosolic localization (Cyt; see upper panel for a representative example), a
dual nuclear/cytosolic localization (Nuc/Cyt; middle panel) or an exclusive nuclear localization (Nuc; lower panel) were counted and expressed as percentage of
ACOX1-Flag-expressing cells in the bar graph. Note that at least 200 cells were analysed per condition. Scale bar, 10 µm.

---

rsob.royalsocietypublishing.org
2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (EHHADH), one of the few monomeric proteins of the peroxisomal matrix [74]. Indeed, studies in a family affected with inherited renal Fanconi’s syndrome revealed that a single missense mutation near the N-terminus of this enzyme was sufficient to create a mitochondrial targeting sequence thus resulting in its mitochondrial mistargeting [75]. Clearly, there is no reason to invoke a defect in the oligomerization of a peroxisomal matrix protein to explain its mistargeting to mitochondria (see also [76] for a discussion on this issue). Rather, the fact that a fraction of both mutant enzymes is missorted to mitochondria suggests that the mutations they harbour not only create mitochondrial targeting information but also interfere with their expedite folding, because only unfolded proteins are accepted by the mitochondrial import machinery [77].

The data presented in this work add to a number of observations suggesting that several newly synthesized peroxisomal matrix proteins arrive at the peroxisomal membrane still as monomers. A particularly interesting finding of our work is that both ACOX1 and UOX, similarly to catalase and sterol carrier protein x [23,61], can be easily obtained in a soluble, monomeric state. The solubility of all these proteins, together with catalase comprise one-third of the total matrix proteins found in rat/mouse liver peroxisomes [62,64].

Interestingly, in vitro import assays revealed that monomeric ACOX1 and UOX are more efficiently imported into peroxisomes than the corresponding oligomeric versions. The results of the co-transfection experiments presented in figure 4, showing that HA-ACOX1 acquires a peroxisomal localization in a much more efficient manner than ACOX1-Flag, are also compatible with this interpretation. While these findings suggest that the PIM displays a preference for monomeric proteins, they do not unveil the mechanistic reasons for such preference. Our in vivo data suggesting that import of dimeric ACOX1 is a low-efficiency process could be explained by simply assuming that interaction of monomeric ACOX1 with PEX5 is a much faster event than the dimerization of the enzyme in the cytosol. However, the in vitro import assays presented here suggest that the preference of the PIM for monomeric proteins may also have other reasons. For instance, it is possible that PEX5 binds monomeric proteins in a faster/stronger manner than it binds the corresponding oligomeric versions. Some data suggesting that this may be the case for catalase have been presented before [61]. Alternatively, the preference of the PIM for monomeric proteins might be exerted by the DTM itself. In this hypothetical scenario, the DTM would accept monomeric proteins having a near-native (more flexible) conformation more efficiently than already oligomerized (rigid) proteins. Discriminating between these possibilities will be a difficult task, requiring much more than the presently available data presented here show that the previously reported capacity of PEX5 to bind monomeric catalase, blocking its oligomerization [61], is also valid for ACOX1 and UOX, two proteins which together with catalase comprise one-third of the total matrix proteins.

Figure 5. Working model for the initial steps of the peroxisomal matrix protein import pathway. Newly synthesized proteins are bound by chaperones during or immediately after their synthesis on cytosolic ribosomes. Successfully folded proteins are then released as near-native monomers and bound by cytosolic PEX5. These monomers are transported to the peroxisomal matrix where oligomerization can occur. Peroxisomal matrix proteins that do not fold correctly (e.g. as a result of mutation) are not released from the chaperones. Retained proteins may be degraded or mistargeted, as is the case of some mutant forms of alanine-glyoxylate amino transferase and 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase which target mitochondria [75,76]. Some proteins may also be imported after the DTM-PTS assembly intermediates, and, on the other hand, that (partial) folding and oligomerization of these monomeric proteins are not obligatory coupled events. Importantly, the
available qualitative data on the protein–protein interactions that govern the peroxisomal protein import pathway.

Although there is still much to be learned on how newly synthesized peroxisomal proteins are transported to the organelle matrix, the data presented here together with a number of previous findings (see Introduction section) support a model in which: (i) many newly synthesized peroxisomal proteins are folded by cytosolic chaperones and released as soluble monomers; (ii) these monomers are then bound by cytosolic PEX5, which blocks their oligomerization; and finally, (iii) these monomeric cargoes are translocated by a single PEX5 molecule into the matrix of the organelle where oligomerization occurs (figure 5).

5. Material and methods

5.1. Plasmids and recombinant proteins

The cDNAs encoding mouse ACOX1 (clone ID 5704873, Open Biosystems) and UOX (clone ID 5136328, Open Biosystems) were amplified by PCR using the primers 5′-GCTAATTCTGAGCATCAAAGCTTCGACTG-3′ and 5′-GGGCCGTGGCTCAGCATCAAAGCTTCGACTG-3′, respectively, and cloned into a XbaI/KpnI digested pGEM4 vector (Promega). For the mammalian expression vector encoding 2HA-ACOX1–3NLS (pOI16), the cDNA encoding mouse ACOX1 was amplified by PCR (template: pGEM4–ACOX1; primers: 5′-AGCTTACCATGGGCTACCCCTATGATGTGCCCGATTACGCCGGAGGGTAC-3′ and 5′-GCCGCGGGTACCAACTACTTATCCTGCGCAAGGGGC-3′), digested with EcoRI/NotI, and ligated into EcoRI-NotI-restricted pCMV-Tag4A (Stratagene). The mammalian expression vector encoding 2HA-ACOX1–3NLS (pOI16) was generated in two steps: first, a plasmid encoding myc-ACOX1–3NLS (pCL5) was constructed by ligating an EcoRI/BglII-restricted PCR fragment (template: pMF1636; primers: 5′-GGGAAATCCAGATGAGCCACCAGTCCGGAAGG-3′ and 5′-GGGAGATCCTCGAGTCGAGGCCGTCGTCCTAACATG-3′) into EcoRI/BglII-restricted CL-Myc-DAO-3NLS (this plasmid was kindly provided by Dr Y. M. Go (Tufts University, MA, USA)); next, the Sall/NotI fragment of pMF1808 was replaced by the Sall/NotI fragment of pCL5. To generate pGEM4–ACOX1-Flag, the corresponding cDNA was amplified by PCR (template: pMF1809; primers: 5′-GCTAATTCTAGACCCACCATGATCCCGGATCATGGCAAGG-3′ and 5′-GCCCCTGACCACCAACTATGACCCCGTGAGGG-3′), digested with EcoRI/NotI and ligated into pCMV-Tag4A (Stratagene). The mammalian expression vector encoding 2HA-ACOX1–3NLS (pO116) was generated

5.2. Synthesis of radiolabelled proteins

To generate the plasmid pGEM4–2HA (Promega), two copies of the haemagglutinin (HA) tag, were annealed and amplified by PCR using the primers 5′-GCTAATTCTAGACCCACCATGATCCCGGATCATGGCAAGG-3′ and 5′-GCCCCTGACCACCAACTATGACCCCGTGAGGG-3′, respectively, and cloned into an XbaI/KpnI digested pGEM4* vector (Promega). To generate the plasmid pGEM4–2HA-ACOX1, digested with EcoRI/NotI, primers 5′-AGCTTACCATGGGCTACCCCTATGATGTGCCCGATTACGCCGGAGGGTAC-3′ and 5′-GCCGCGGGTACCAACTACTTATCCTGCGCAAGGGGC-3′, respectively, and cloned into an XbaI/KpnI-restricted pGEM4 vector. The correctness of all plasmids was confirmed by DNA sequence analysis (LGC Genomics). The recombinant large isoform of mouse PEX5 [61], a protein comprising amino acid residues 315–639 of PEX5 (TPRs [78]), and TPRs containing the missense mutation N526K (TPRs [N526K], numbering of full-length PEX5 [79]) were obtained as previously described.

5.3. Immunoprecipitations

Radiolabelled proteins synthesized in the presence of 1 μM recombinant PEX5 were diluted to 500 μl with buffer A (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA–NaOH, pH 8.0, 10% (w/v) glycerol, 0.1% (w/v) Triton X-100) supplemented with 0.025% of bovine serum albumin (BSA) and 1:500 (v/v) mammalian protease inhibitor mixture (Sigma). The composition of translation product mixtures, prepared in the presence and absence of PEX5, was made identical by adding recombinant PEX5. Immunoprecipitation was done using 30 μl of anti-HA antibody agarose beads (Sigma) for 3 h at 4°C. Beads were washed four times with 150 μl of buffer A. Immunoprecipitated proteins were analysed by SDS-PAGE/autoradiography.

5.4. Sucrose gradients

Radiolabelled proteins were incubated for 5 min at room temperature in the presence or absence of 1 μM of either PEX5 or a control protein (soybean trypsin inhibitor), in 200 μl of buffer B (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA–NaOH, pH 8.0 and 1 mM DTT). The mixtures were then loaded onto the top of a continuous 5–30% (w/v) sucrose gradient in the same buffer (generated using 5.4. Sucrose gradients

Radiolabelled proteins were incubated for 5 min at room temperature in the presence or absence of 1 μM of either PEX5 or a control protein (soybean trypsin inhibitor), in 200 μl of buffer B (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA–NaOH, pH 8.0 and 1 mM DTT). The mixtures were then loaded onto the top of a continuous 5–30% (w/v) sucrose gradient in the same buffer (generated using 107ip GRADIENT MASTERTM; BioComp, Canada) and subsequently centrifuged at 39 000 r.p.m. for 29 h at 4°C. Beads were washed four times with 150 μl of buffer A. Immunoprecipitated proteins were analysed by SDS-PAGE/autoradiography.
autoradiography analyses were done as described [36]. Mouse liver peroxisomal matrix proteins were obtained by sonicating purified organelles (800 μg of protein) in buffer B supplemented with 1:500 (v/v) mammalian protease inhibitor mixture (Sigma) followed by centrifugation for 30 min at 100 000 g. The supernatant was loaded onto the top of a sucrose gradient and centrifuged, as above.

5.5. In vitro import reactions

Rat liver PNS was prepared as described before [22]. In vitro import assays containing 500 μg of PNS and the radio-labelled protein were incubated for 45 min at 37◦C, in 100 μl of import buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.4, 50 mM KCl, 3 mM MgCl2, 20 μM methionine, 2 μg ml−1 N-(trans-epoxysuccinyl)-l-leucine 4-guanidino butylamide) containing 3 mM ATP, 2 mM glutathione, and recombinant PEX5 (1.5 nM for ACOX1 and 7.5 nM for UOX). Where indicated, TPRs or TPRs(N526K) (0.3 μM final concentration) was also added to assays. Note that incubation of radio-labelled ACOX1 and UOX with recombinant PEX5 before proceeding with the import assay, a strategy used before for sterol carrier protein x [23], resulted in only a modest increase in the import yields of ACOX1 and UOX (approx. 1.5-fold increase in 45 min import reactions). Thus, for practical reasons, this step was not included in the experiments described here. It is likely that the half-lives of the PEX5–ACOX1 and PEX5–UOX protein complexes are too short to benefit from this step, although further data are necessary to confirm this possibility. Protease treatment of import reactions was done using 400 μg ml−1 of proteinase K (final concentration) for 40 min, on ice. After protease inactivation with phenylmethysulfonyl fluoride (500 μM final concentration) was also added to assays. Note that incubation of radiolabelled ACOX1 and UOX with recombinant PEX5 (1.5 nM for ACOX1 and 7.5 nM for UOX). Where indicated, TPRs or TPRs(N526K) (0.3 μM final concentration) was also added to assays. Note that incubation of radiolabelled ACOX1 and UOX with recombinant PEX5 before proceeding with the import assay, a strategy used before for sterol carrier protein x [23], resulted in only a modest increase in the import yields of ACOX1 and UOX (approx. 1.5-fold increase in 45 min import reactions). Thus, for practical reasons, this step was not included in the experiments described here. It is likely that the half-lives of the PEX5–ACOX1 and PEX5–UOX protein complexes are too short to benefit from this step, although further data are necessary to confirm this possibility. Protease treatment of import reactions was done using 400 μg ml−1 of proteinase K (final concentration) for 40 min, on ice. After protease inactivation with phenylmethysulfonyl fluoride (500 μg ml−1, final concentration) for 2 min on ice, organelles were isolated by centrifugation and analysed as described before [28].

5.6. Cell culture, transfections and immunofluorescence microscopy

COS-7 cells (kindly provided by Dr M. Schrader (University of Exeter, UK)) were cultured at 37◦C in a humidified 5% CO2 incubator in minimum essential medium Eagle α (Lonza) supplemented with 10% (v/v) fetal bovine serum superior (Biochrom AG), 2 mM Ultraglutamine-1 (Lonza) and 0.2% (v/v) Mycozap (Lonza). Cells were transfected using Invitrogen’s Neon Transfection System (1050 V, 30 ms pulse width, 1 pulse). Samples for immunofluorescence microscopy were fixed and processed as described before [80]. The rabbit polyclonal antiserum against human PEX14 has been described elsewhere [81]. DAPI (Roche), the mouse anti-HA (Sigma) and anti-FLAG (Stratagene) antibodies, and the Alexa Fluor 488- (Invitrogen) or Texas Red- (Calbiochem) conjugated secondary antibodies were commercially obtained. Fluorescence was evaluated on a motorized inverted IX-81 microscope (Olympus) controlled by Cell-M software (Olympus). The technical specifications of the objectives, excitation and emission filters, and digital camera have been described elsewhere [82]. Fluorescence intensity values were obtained using [MACS] software.

5.7. Miscellaneous

Isolation of highly pure peroxisomes from mouse liver by differential centrifugation and Nycodenz gradient purification was performed as described [83,84]. The antibodies directed to the 21 kDa and 53 kDa fragments of ACOX1 have been described before [85]. For the protease susceptibility assays, 35S-labelled proteins isolated from a sucrose gradient and native ACOX1 (from mouse liver purified peroxisomes) were diluted in import buffer and subjected to proteinase K digestion (10–400 μg ml−1, final concentration) in the presence or absence of 1% (w/v) Triton X-100, as specified, and incubated for 40 min on ice. After protease inactivation with phenylmethysulfonyl fluoride, proteins were precipitated with trichloroacetic acid and processed as previously described [22].

Acknowledgements. We thank Dr O. Apanasets (KU Leuven, Belgium) for her help with the in cellulo studies.

Funding statement. This work was funded by Fundo Europeu de Desenvolvimento Regional (FEDER) through the Operational Competitiveness Programme (COMPETE); by National Funds through Fundação para a Ciência e a Tecnologia (FCT) under the project FCOMP-01–0124-FEDER-022718 (PEst-C/SAU/LA0002/2010) and FCOMP-01–0124-FEDER-019731 (PTDC/BIABCM/118577/2010); by Portuguese National Mass Spectrometry Network (RNEM) through the project REDE/1504/REM/2005; and by Quimica Orgánica, Produtos Naturais e Agroalimentares (QOPNA) research unit funds provided by FCT, European Union, QREN, FEDER and COMPETE under the projects PEst-C/QUI/UI0082/2013 and FCOMP-01–0124-FEDER-037296. M.O.F., T.F., T.A.R., M.P.P. and C.P.G. were supported by FCT, Programa Operacional Potencial Humano (POPH) do Quadro de Referência Estratégico Nacional (QREN) and Fundo Social Europeu. The work done in Leuven was supported by grants from the ‘Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Onderzoeksproject G.0754.09)’ and the KU Leuven (OT/14/100).


Conflict of interests. The authors declare no competing interests.

References


43. Lee MS, Mullen RT, Trelease RN. 1997 Oilsed isocitrate lyases lacking their essential type 1 peroxisomal targeting signal are piggybacked to glyoxosomes. Plant Cell 9, 185 – 197. (doi:10.1105/tpc.9.2.185)


