Alcohol oxidase and dihydroxyacetone synthase, the abundant peroxisomal proteins of methylotrophic yeasts, assemble in different cellular compartments

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SUMMARY

Alcohol oxidase (AO) and dihydroxyacetone synthase (DHAS) constitute the bulk of matrix proteins in methylotrophic yeasts, model organisms for the study of peroxisomal assembly. Both are homooligomers; AO is a flavin-containing octamer, whereas DHAS is a thiamine pyrophosphate-containing dimer. Experiments in recent years have demonstrated that assembly of peroxisomal oligomers can occur before import; indeed the absence of chaperones within the peroxisomal matrix calls into question the ability of this compartment to assemble proteins at all. We have taken a direct pulse-chase approach to monitor import and assembly of the two major proteins of peroxisomes in Candida boidinii. Oligomers of AO are not observed in the cytosol, consistent with the proteins inability to undergo piggyback import. Indeed, oligomerization of AO can be followed within the peroxisomal matrix, directly demonstrating the capacity of this compartment for protein assembly. By contrast, DHAS quickly dimerizes in the cytosol before import. Binding and import was slowed at 15°C; the effect on AO was more dramatic. In conclusion, our data indicate that peroxisomes assemble AO in the matrix, while DHAS undergoes dimerization prior to import.

Key words: Peroxisomes, Microbodies, Protein trafficking, Membrane translocation, Chaperones

INTRODUCTION

Peroxisomal protein import has been the subject of fruitful research in the past several years, and as a result many steps in this process have been identified (Hettema et al., 1999; Kunau, 1998; Subramani et al., 2000). One of two peroxisomal targeting signals, PTS1 and PTS2, allows proteins destined to peroxisomes to bind to cytosolic receptors (Pex5p or Pex7p for PTS1 and PTS2, respectively). The substrate-receptor pair then binds to a docking complex (containing at least Pex13p, Pex14p and Pex17p) on the peroxisomal membrane. Several other membrane-associated proteins function downstream in a way that is ill-defined at present to promote translocation of the substrate. The medical significance of peroxisomal protein import is demonstrated by the peroxisomal deficiency diseases Zellweger syndrome and neonatal adrenoleukodystrophy, in which there is a global failure of peroxisomal matrix protein import (Fujiki, 1997).

While most organelles that undergo protein import accept only unfolded substrates, several lines of evidence indicate that peroxisomes import folded proteins and oligomers: (1) monomers of oligomeric peroxisomal proteins and carriers that lack a PTS can be imported if coexpressed with partners containing a PTS (Elgersma et al., 1996; Glover et al., 1994; McNew and Goodman, 1994); (2) microinjected protein aggregates and even colloidal gold particles that contain a PTS can be imported (Walton et al., 1995); (3) aminopterin does not inhibit the import of dihydrofolate reductase into peroxisomes (Hausler et al., 1996); (4) mistargeting of alanine:glyoxylate aminotransferase to mitochondria in the disease human primary hyperoxaluria type I requires mutations that both generate a mitochondrial targeting signal and disrupt dimerization (Leiper et al., 1996); (5) in vitro import of protein dimers has been demonstrated (Brickner et al., 1997); and (6) the PTS receptors Pex5p and Pex7p can be found within the peroxisomal matrix suggesting that they accompany substrate into this compartment and then recycle back to the cytosol (Dodt and Gould, 1996; Szilard et al., 1995; Zhang and Lazarow, 1995).

Recent evidence has shown that peroxisomes are not unique in their ability to import folded proteins. The cvt pathway for the import of cytosolic precursors into the yeast vacuole involves the engulfment of cytosolic oligomers by membrane and fusion to the vacuole (Klionsky and Ohsumi, 1999). Thylakoid membranes of plants can export proteins from the stroma through three different pathways, one of them (the ΔpH pathway) accepts folded precursors as substrates (Creighton et al., 1995). A similar mechanism of export, the twin-arginine translocation pathway, is found in Escherichia coli (Hunds et al., 1998).

While many experiments have clearly shown that peroxisomes can import oligomers, questions remain about the physiological importance of this mechanism. If a protein can be imported into peroxisomes as an oligomer, does it normally utilize that pathway? Is oligomeric import the exclusive pathway for peroxisomes? Since classical chaperones have not been found within the organelle, do peroxisomes have the
capacity to assemble monomers into active oligomers, and do they ever do so?

To answer these questions we used a pulse-chase approach in the yeast *Candida boidinii* to ascertain the relationship between peroxisomal import and oligomerization. Methylo trophic yeasts such as *C. boidinii*, when cultured on methanol, contain large peroxisomes that consist almost exclusively of two oligomeric proteins, alcohol oxidase (AO), an octamer, and dihydroxyacetone synthase (DHAS), a dimer (Goodman, 1985; Veenhuis and Harder, 1987). We now report that AO is imported into peroxisomes over several minutes as a monomer, consistent with a previous finding that AO lacking a PTS could not be imported with subunits containing a PTS (Waterham et al., 1997). Octamerization of AO is demonstrated to occur in the peroxisomal matrix. By contrast, DHAS quickly dimerizes in the cytosol prior to its import.

**MATERIALS AND METHODS**

**Cell culture and generation of activated spheroplasts**

*Candida boidinii* was cultured in mineral medium (Sahm and Wagner, 1972) supplemented with 1% MeOH and 0.5 M MgSO4. Addition of 0.5 M MgSO4 to the culture medium was found to yield more reproducibility in labeling and expression of peroxisomal proteins during metabolic labeling. All of the following steps to generate and activate spheroplasts were performed at 30°C with pre-warmed reagents and incubations in a rotary shaker. 100 ml of cell culture was centrifuged at 1 OD600/ml was harvested by centrifugation at 6,750 g for 10 minutes, resuspended in 30 ml H2O, and repelled. Cells were resuspended in 10 ml 0.1 M Tris-SO4 (pH 9.3) with 10 mM DTT, and incubated for 10 minutes. Cells were harvested and washed in 30 ml 0.5 M MgSO4. The pellet was resuspended in 10 ml 20 mM KH2PO4 pH 7.5 with 0.5 M MgSO4 and 1% MeOH, and then 15 μg of 100T Zymolyase was added. Spheroplasting was allowed to occur for approximately 45-60 minutes until conversion to spheroplasts was complete, as monitored microscopically by the ability of added H2O to lyse a large majority of the cells. 20 ml 0.5 M MgSO4 was then added, the cells were pelleted at 5,820 g for 10 minutes and then resuspended in 10 ml mineral medium with MeOH and MgSO4 (identical to culture medium) and incubated at 30°C for 1 hour to activate prior to labeling.

**Metabolic labeling and organelle fractionation**

Activated spheroplasts were radiolabeled for 5 minutes with 30 μCi 35S-methionine (New England Nuclear Life Science Products, Boston, MA) per 500 μl spheroplasts. Unlabeled methionine/cysteine, a solution of 250 mM each, was then added to a final concentration of 10 mM each for the chase, and cells were allowed to incubate for the times indicated. At each time point of chase, 200 μl of cell suspension was quickly pipetted into 1 ml of pre-chilled 1 M sorbitol on ice. (For the temperature shift experiments, the 15 degree shift was performed in a water bath using small volumes of cell suspension (less than 1 ml) and in thin plastic tubes to ensure rapid temperature equilibration.) Cells were harvested by centrifugation at 5,000 g spin for 5 minutes at 4°C in a microfuge. They were then resuspended in 200 μl 1 M SMA (1 M sorbitol, 5 mM MES (2-[N-morpholino]ethanesulfonic acid) pH 5.5, 0.2 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), then lysed by the addition of 800 μl 0.25 M (referring to sorbitol concentration) SMA. The lysate was vortexed three times for 10 seconds with a 5 minute incubation on ice in between each vortexing. Separation of cytosol (SUP) and organellar pellet (PEL) was performed by centrifugation at 20,000 g (in a microfuge) for 15 minutes at 4°C. For experiments in which the PEL was further fractionated, a duplicate PEL fraction was resuspended in 500 μl 30 mM Tris-HCl pH 9.0 with 5 mM EDTA to lyse peroxisomes contained therein. Separation of membrane (MEM) and matrix (MAT) fractions was then performed by a single centrifugation at 100,000 g for 15 minutes at 4°C in a Beckman TL-100 rotor. Immunoprecipitation of AO and DHAS was performed on each fraction using antibodies to the corresponding proteins (see Reagents and other methods). Radiolabel was visualized by fluorography and the dried gel was exposed to BioMax single emulsion film (Eastman Kodak, Rochester, NY).

**Analysis of oligomeric state of AO and DHAS**

The oligomeric state of AO contained in SUP and MAT fractions was determined by velocity sedimentation in sucrose gradients and immunoprecipitation of gradient fractions as previously reported (Goodman et al., 1984). A similar procedure was performed to establish the oligomeric state of DHAS except gradients were centrifuged for 12 hours instead of 3.5 hours. Bovine serum albumin (BSA), mature DHAS and AO were used as molecular mass standards in the gradients and visualized by Coomassie Blue staining of SDS-PAGE gels.

**Reagents and other methods**

Polyclonal antibodies for AO (Goodman et al., 1984) and DHAS (Goodman, 1985), and a monoclonal antibody against Pmp47 (Goodman et al., 1986) used in immunoblots, have been previously described. Immunoblots were performed with Enhanced Chemiluminescence reagents as directed by the manufacturer (Amersham Life Science, Arlingon Heights, IL). SDS-PAGE (Laemmli, 1970) was performed with 9% gels and a separating gel of pH 9.2. Formaldehyde dehydrogenase was assayed enzymatically (Egli et al., 1980).

**RESULTS**

Analysis of peroxisomal targeting intermediates required a fractionation procedure to quickly, cleanly and reproducibly separate cytosol from peroxisomal matrix and membrane in high yield following pulse-chase. Such a method is outlined in Fig. 1A. Following pulse-chase of activated spheroplasts, cells were quickly lysed and subjected to a single centrifugation to separate cytosol (and light membranes, the SUP fraction) from peroxisomes (and other organelles, the PEL fraction). Fig. 1B demonstrates that this step results in the release of 94% of a cytosolic marker, formaldehyde dehydrogenase, a measure of the high efficiency of spheroplast lysis. Integrity of peroxisomes can be assessed in Fig. 1C, illustrating a protein stain of the fractions after SDS-PAGE. Alcohol oxidase and dihydroxyacetone synthase are the most abundant proteins in the cell, an indication of the massive proliferation of peroxisomes when cells are incubated in methanol medium (Goodman et al., 1984; Sahm et al., 1975). Very few of these matrix proteins are seen in the SUP fraction compared with the PEL fraction. Densitometry of these bands reveals at least 80% of these markers fractionate in the PEL (AO is generally above 90%), indicating that almost all peroxisomes are intact. Fig. 1D, illustrating an immunoblot against the integral peroxisomal membrane protein Pmp47 (Goodman et al., 1986), shows that all of the peroxisomal membrane fractionates with the PEL fractions.

To release matrix from the PEL fraction, the pH was raised to 9.0 (Goodman et al., 1984), and the sample was subjected to high speed centrifugation (Fig. 1A). Virtually all the AO and DHAS bands remained in the supernatant (MAT, Fig. 1C), while Pmp47 was recovered only in the pellet (MEM, Fig. 1D).
The data show that the matrix fraction contained virtually all
the AO and DHAS from the pellet (Fig. 1C) and no
peroxisomal membrane (Fig. 1D). In summary, there is little
cross contamination between cytosol (in the SUP), matrix and
peroxisomal membrane.

Using pulse-chase and this fractionation protocol, we
followed the trafficking of newly synthesized AO and DHAS,
and the results up to 15 minutes of chase are shown in Fig.
2A,B. Alcohol oxidase chases from the cytosol (SUP) to
peroxisomes (PEL) as has been shown previously in a more
detailed analysis (Goodman et al., 1984). A similar pattern was
observed for DHAS, although the kinetics of association with
peroxisomes was considerably faster (Goodman, 1985). The
fractions of AO and DHAS found in the SUP could not be
sedimented at 100,000 g, indicating that they are in the cytosol
(data not shown). During this time course both proteins
appeared in the peroxisomal matrix. We always saw a
lag in appearance of both labeled proteins between the
PEL and in the MAT (Fig. 2A). Very little AO
or DHAS was seen in the MEM fraction at any
time point. This loss suggested that the
immunoprecipitation from the membrane fraction was
ineffective. When the fractions were visualized
directly without immunoprecipitation (Fig. 2C), there
was clearly a significant amount of newly synthesized
AO and DHAS associated with the membrane fraction
at early time points. In fact, more AO precursor is
observed in the membrane than the matrix at 0 and 1
minutes.

The oligomeric state of AO in the cytosol and
matrix, as determined by velocity sedimentation, as a
function of time after synthesis, is shown in Fig. 3. AO
is a monomer in the cytosol at all time points
examined, suggesting that oligomerization does not
happen in this compartment. As AO appears in the
matrix (1 minute chase), it is mostly monomer. Most
of the monomer that we detect in the matrix is very
sensitive to proteolysis during sample processing,
much more so than monomers in the cytosol, and is
clipped to a stable intermediate (indicated by asterisks
in Fig. 3). We have been unable to fully prevent this
degradation with protease inhibitors. At longer chase
times, octamer accumulates in the matrix, while
monomers still enter peroxisomes (15 minutes). In
some experiments AO of an intermediate size is seen
(15 minute point, open arrowhead, more apparent at
lower temperatures), which may indicate dimeric
or tetrameric forms. This experiment demonstrates
that the peroxisomal matrix has the capacity to
oligomerize AO.

Parallel fractions from the same experiment were
centrifuged in the sucrose gradients for longer times
to separate monomeric from dimeric DHAS (Fig. 4).
In contrast to AO, DHAS clearly dimerizes in the
cytosol prior to import. Even at 0 minutes of chase
about half of DHAS is already a dimer; the ratio
increases further with longer chase. Dimeric DHAS is
seen in the matrix, even at the earliest time points.
Considering the efficient dimerization of DHAS in the
cytosol and the appearance of dimer in the matrix at
the earliest time point, the simplest interpretation of
our data is that the dimeric form of DHAS is the major if not
the only species that crosses the peroxisomal membrane.

Having shown that AO is imported as a monomer and DHAS
as a dimer, we next performed import experiments at lower
temperatures to attempt to uncover mechanistic differences in
the import of the two substrates. At 15°C, the association of
both AO and DHAS with peroxisomes revealed a complex
triphasic kinetic pattern with AO exhibiting slower kinetics in
all three phases. The slower import kinetics of AO were not
attributable to a decrease in the rate of octamerization in the
matrix (data not shown). Although AO appears to cross the
peroxisomal membrane more slowly than DHAS, our data do
not allow us to conclude that mechanistic differences exist
between the import of these two substrates.

In summary, we have been able to monitor simultaneously
the import and assembly of endogenous peroxisomal proteins

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**Fig. 1.** Separation of peroxisomal subcompartments from activated
spheroplasts. (A) Outline of procedure. After metabolic labeling, activated
spheroplasts are subjected to osmotic lysis at pH 5.5. An organellar pellet (PEL)
and supernatant (SUP) are generated by centrifugation of the lysate at 20,000 g.
(There are no prespins.) The pellet is resuspended in buffer at pH 9.0 to lyse
peroxisomes, and then spun at 100,000 g, generating a supernatant containing
peroxisomal matrix (MAT) and a pellet containing peroxisomal membrane
(MEM). Analysis of markers in a typical experiment is shown in B-D.
(B) Formaldehyde dehydrogenase, a cytosolic enzyme. There is little
contamination of cytosol in the matrix. (C) Coomassie Blue staining indicates
the integrity of peroxisomes after cell lysis, and the subsequent release of
matrix. (D) Immunoblot with anti-Pmp47, an integral peroxisomal membrane
protein.
Our data show that AO enters peroxisomes as monomers and that the peroxisomal matrix has the capacity to assemble octamers from this precursor. By contrast, DHAS dimers assemble quickly in the cytosol before translocation into the matrix.

DISCUSSION

To examine the physiological significance of oligomeric import and determine whether matrix is capable of forming oligomeric proteins, we undertook a pulse-chase approach to directly observe these processes. We chose AO and DHAS from C. boidinii because each of these proteins comprise over 10% of cell protein and are very easy to detect. Our pulse-chase results depended on technical improvements over previous work in C. boidinii (Goodman, 1985; Goodman et al., 1984). Cell growth and spheroplasting conditions were adjusted to yield efficient and reproducible radiolabeling and complete cell lysis while keeping peroxisomes intact. This procedure obviated the need to discard a low-speed pellet, which can contain most of the cellular organelle mass (data not shown), thus skewing the correct ratio of substrates in cytosol versus in organelles. The subsequent lysis of the peroxisomal membrane by raising the pH allowed a fast separation of cytosol, peroxisomal membrane and matrix. This procedure was then coupled with sucrose velocity sedimentation to determine the compartment in which assembly of oligomers occurred.

These observations are novel in two ways. First, this is the only direct demonstration to our knowledge that the peroxisomal matrix can support the formation of quaternary structure. Our results suggest that the import of microinjected AO octamers into peroxisomes does not reflect the physiological import mechanism for these substrates (Walton, 1996). Once AO monomers cross the membrane, an intermediate can be detected that migrates between monomer and octamer in sucrose velocity gradients. It is not clear whether this oligomeric form represents homomers or heteromers. Why does oligomerization of AO occur in the matrix and not the cytosol? There are three possibilities, which are not mutually exclusive: (1) conditions within the matrix are favorable for spontaneous assembly of AO octamers (an optimal pH or specific ion concentration, for example); (2) proteins within the matrix catalyze assembly into octamers; or (3) molecular chaperones within the cytosol prevent assembly. Chaperones may not be required for octamer formation since monomers that have been dissociated from holoenzyme by treatment with glycerol can spontaneously reassemble into active enzyme (Boteva et al., 1999). However, dissociation is irreversible once monomers are sufficiently denatured to release cofactor. Does the monomeric form that is imported into peroxisomes contain FAD? Riboflavin starvation in the yeast Hansenula polymorpha leads to inefficient import of AO, suggesting that binding to FAD normally precedes import (Evers et al., 1994). Nevertheless, data is also available to suggest involvement of chaperones in the assembly process. Cytosolic hsp70 is known to keep proteins unfolded and competent for import into mitochondria and endoplasmic reticulum (Deshaies et al., 1988) and could play a similar role in preventing AO assembly in the cytosol. There is also evidence for the involvement of matrix chaperones: mutants in H. polymorpha that fail to generate peroxisomes assemble AO in the cytosol, as if folding factors found in the matrix in wild-type cells catalyze this process in the cytosol of the mutants.

in C. boidinii. Our data show that AO enters peroxisomes as monomers and that the peroxisomal matrix has the capacity to assemble octamers from this precursor. By contrast, DHAS

Fig. 2. The import of AO and DHAS from cytosol into peroxisomal matrix. Activated spheroplasts were labeled as described and chased for the times indicated. Spheroplasts from each time point were subjected to fractionation. (A) Fractions were immunoprecipitated with antibody to the proteins indicated and subjected to SDS-PAGE and fluorography. (B) Immunoprecipitates were quantified by densitometry. Results were expressed as a ratio of the label in the pellet compared with supernatant and pellet. (C) Sample buffer was added directly to the PEL, MAT and MEM fractions before SDS-PAGE and fluorography.
Assembly of AO and DHAS

We have attempted to form oligomers by incubating metabolically pulse-labeled monomers with concentrated matrix but have not yet been successful since we have been unable to control the action of a protease activity to which monomeric AO is particularly sensitive (data not shown). This activity is apparent in Figs 3, 5.

Fig. 3. Alcohol oxidase is imported as a monomer. The supernatant and matrix fractions from pulse-chased spheroplasts were subjected to velocity sedimentation. Fractions of the sucrose gradients were immunoprecipitated with anti-AO antiserum. BSA and mature AO were used as markers of the monomeric and octameric species. Asterisk represents an AO degradation product. Open arrowhead indicates intermediate AO forms on gradient.

Fig. 4. Dihydroxyacetone synthase is imported as a dimer. Analysis similar to that in Fig. 3 except the gradients were spun for a longer time and fractions were subjected to immunoprecipitation with anti-DHAS antiserum. BSA and mature DHAS served as markers of monomeric and dimeric species.

Second, this report is the first study in which the extent to which a protein oligomerizes prior to peroxisomal import has been determined. Our data show that DHAS extensively and perhaps completely dimerizes prior to import since very little if any monomeric DHAS is detected in the peroxisomal matrix. These data suggest that oligomeric import is the normal mechanism for translocation of this substrate, unlike AO. Since our results are based on kinetic analysis alone, we cannot rule out the possibility that DHAS binds to the membrane as a dimer, quickly dissociates into monomers, and reassembles into dimers on the matrix side of the membrane following translocation. This pathway appears very unlikely. The only known chaperones that have the ability to dissociate and unfold proteins and protein aggregates are those in the Hsp100 family, and studies in vivo and in vitro have shown that this process requires several minutes (Glover and Lindquist, 1998); we see no such lag in our import reactions, we do not detect a buildup of DHAS in the membrane prior to import, and the amount of monomers at any time in the matrix is negligible. Certainly the simplest interpretation of our data is that the dimeric form itself is imported.

The experiments at low temperature were an attempt to kinetically dissect the import process of AO and DHAS. Import is slowed for both substrates but especially for AO. Further experiments in a more genetically manipulatable system may allow us to pinpoint the rate limiting step of the import process at a molecular level.

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REFERENCES

