Redirection of peroxisomal alcohol oxidase of *Hansenula polymorpha* to the secretory pathway

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**Abstract**

We report on the rerouting of peroxisomal alcohol oxidase (AO) to the secretory pathway of *Hansenula polymorpha*. Using the leader sequence of the *Saccharomyces cerevisiae* mating factor a (MFa) as sorting signal, AO was correctly sorted to the endoplasmic reticulum (ER), which strongly proliferated in these cells. The MFa presequence, but not the prosequence, was cleaved from the protein. AO protein was present in the ER as monomers that lacked FAD, and hence was enzymatically inactive. Furthermore, the recombinant AO protein was subject to gradual degradation, possibly because the protein did not fold properly. However, when the *S. cerevisiae* invertase signal sequence (ISS) was used, secretion of AO protein was observed in conjunction with bulk of the protein being localized to the ER. The amount of secreted AO protein increased with increasing copy numbers of the AO expression cassette integrated into the genome. The secreted AO protein was correctly processed and displayed enzyme activity.

**Introduction**

The yeast *Hansenula polymorpha* is able to grow on methanol as the sole carbon and energy source. Growth on this compound is associated with induction of the synthesis of a specific set of proteins, which catalyze the conversion of methanol into biomass and energy (for a recent review, see Van der Klei et al., 2006). Alcohol oxidase (AO) is the first enzyme of methanol metabolism, and catalyzes the oxidation of methanol to formaldehyde and hydrogen peroxide. The enzymatically active form of AO is a homo-octamer of c. 600 kDa. Each subunit contains one FAD molecule as the cofactor (Ozimek et al., 2005). AO is a peroxisomal protein, and is sorted to peroxisomes in a Pex5p-dependent manner (Ozimek et al., 2005).

During the past few years, methylotrophic yeast species, including *H. polymorpha*, have been recognized as attractive hosts for the synthesis and secretion of heterologous proteins (Gellissen & Melber, 1996; Gellissen et al., 2005). Many proteins have been successfully produced on an industrial scale; a prominent example is a hepatitis B antigen that is produced in huge amounts in *H. polymorpha* for use in human healthcare (Hollenberg & Gellissen, 1997).

Oxidoreductases (such as AO) represent a class of proteins of strong scientific and industrial interest. In this study, we have used AO to investigate whether secretion of the enzymatically active enzyme via the secretory pathway could be achieved. The results are included in this article.

**Materials and methods**

**Organisms and growth conditions**

All *H. polymorpha* strains used are derivatives of NCYC495 (Gleeson & Sudbery, 1988). Wild-type (WT) strains (NCYC495 leu1.1 ura3 and NCYC495 leu1.1), WT::P<sub>AOX</sub> MP<sub>zAOX</sub>Δ, Δ<sub>xox</sub>::P<sub>AOX</sub>MP<sub>zAOX</sub>Δpex3 (Baerends et al., 1996), Δ<sub>xox</sub>::P<sub>AOX</sub>MP<sub>zAOX</sub>/Δpex3, WT::P<sub>AOX</sub>ΔISSAOX with various copy numbers and Δ<sub>xox</sub>::P<sub>AOX</sub>ΔISSAOX were grown at 37 °C in: (1) rich complex medium (YPD) containing 1% yeast extract, 2% peptone, and 1% glucose; (2) mineral medium (MM) as previously described (Van Dijken et al., 1976); or (3) YNB without amino acids containing 0.67% Yeast Nitrogen Base (Difco). The carbon sources used were 0.5% glucose, 0.5% methanol or 0.1% glycerol/0.5% methanol; 0.25% ammonium sulfate was used as the nitrogen source 0.25%. Cultivation in continuous culture was performed at 37 °C in MM (Van Dijken et al., 1976) supplemented with 0.2% glucose and 0.25% methanol at a dilution...
rate of 0.1 h⁻¹. Leucine and/or uracil (30 µg mL⁻¹) or riboflavin (1 mM) were added when required.

*Escherichia coli* DH5α (Gibco-Brl, Gaithesburg, MD) was grown at 37 °C in Luria–Bertani medium, supplemented with ampicillin (100 µg mL⁻¹), kanamycin (50 µg mL⁻¹) or erythromycin (150 µg mL⁻¹) when required.

**Miscellaneous DNA techniques**

Standard recombinant DNA techniques, *E. coli* transformation and plasmid isolation procedures were performed essentially as previously described (Sambrook *et al.*, 1989). *Hansenula polymorpha* was transformed by electroporation (Faber *et al.*, 1994b).

**Construction of plasmids containing endoplasmic reticulum (ER)-targeted *H. polymorpha* AOX**

The plasmid containing the *Saccharomyces cerevisiae* mating factor α (MFα) leader sequence fused in-frame to the *H. polymorpha AOX* gene under control of the AO promoter (Pₐₒₓ) was constructed as follows. A 1.8-kb fragment containing the Pₐₒₓ and MFα was isolated by PCR with the MFα primer (5′-GGG GGA TCC TCT TTT ATC CAA GCT GCT TAG CCC) and the M13/pUC universal sequencing primer. The amplified fragment was digested with StuI and BamHI and cloned into pHIPX4-MFα-PAS3 (Kiel *et al.*, 1995) digested with StuI and BamHI, resulting in plasmid pHIPX4-MFα-PAS3. The AOX gene was amplified using PCR and the AOXstart primer (5′-CCC GGA TCC GGC ATT CCT GAC GAA TTC G) and the AOXstop primer (5′-CCG GGG TTA GAA TCT GGC AAG TCC GGT CTC G). Finally, the amplified AOX fragment was digested with BamHI and Smal and cloned into BamHI/Smal-digested pHIPX4-MFα-PAS3, thereby replacing the PAS3 gene. The plasmid pHIPX4-MFα-AOX was used to transformNCYC495 leu1.1 cells. Correct integration into the Pₐₒₓ locus in multiple copies was confirmed by Southern blot analysis (data not shown).

The fragment used to replace the endogenous AOX gene was constructed as follows. A 3.9-kb StuI/Nar1 fragment from pHIPX4-ISSAOX was cloned into pHIPX1 digested with the same enzymes. The resulting plasmid was digested with BamHI, and the 8.6-kb fragment obtained was used to transformNCYC 495 leu1.1 cells. Correct integration into the Pₐₒₓ locus and replacement of the chromosomal AOX gene was confirmed by Southern blot analysis (data not shown). The resulting strain was designated AOΔ::Pₐₒₓ ISS-AOX.

**Biochemical methods**

Cell extracts were prepared as described previously (Van der Heide *et al.*, 2002). Separation of monomeric and octameric AO was performed by sucrose gradient centrifugation (Evers *et al.*, 1996). FAD was measured spectroscopically (Boteva *et al.*, 1999). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and determination of AO activity followed established procedures. Protein concentrations were determined using the Biorad bovine serum albumin as a standard.

Deglycosylation using endoglycosidase H (endo H) (Boehringer) was performed as follows. A volume of a yeast culture corresponding to three OD₆₀₀nm units (volume × OD₆₀₀nm) was harvested by centrifugation. The cell pellet was resuspended in a 12.5% (w/v) trichloroacetic acid and frozen at −80 °C. After thawing, cells were collected by centrifugation at room temperature. The cell pellet was washed twice with ice-cold 80% acetone, and subsequently dissolved in 80 µL of 0.1 M NaOH/1% sodium dodecyl sulfate (SDS)/1% β-mercaptoethanol. After a clarifying spin, the cell extract was boiled for 5 min. For endo H treatment, 15 µL of the extract was added to 35 µL of endo H.
buffer (80 mM sodium acetate, pH 4.5, 0.8% SDS, 1% β-mercaptoethanol) containing 2 μM of endo H (Boehringer) and protease inhibitors, and incubated for 2 h at 37°C. Finally, SDS sample buffer was added, and the samples were boiled for 5 min prior to SDS-PAGE and Western blotting.

Inhibition of glycosylation by tunicamycin was obtained as follows. WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> cells were grown in methanol containing medium for 12 h. Then (t = 0 h), the culture was divided into two halves. To one half, tunicamycin (15 μg mL<sup>−1</sup>) was added; the other half of the culture was used as a control. Samples, taken at t = 0 and 1 h, were analyzed by Western blotting using α-AO antibodies. The relative AO protein levels were determined by densitometry scanning of the blots.

**Electron microscopy**

Whole cells were fixed and embedded in Epon 812 or Unicryl as described previously (Waterham et al., 1994). Ultrathin Unicryl sections were labeled using polyclonal antibodies against various proteins and goat anti-rabbit antibodies conjugated to gold, according to the instructions of the manufacturer (Amersham, UK). Cytochemical localization of AO activity was performed as previously described (Veenhuis et al., 1976).

**Results**

**Peroxisomal AO protein can enter the secretory pathway**

In order to direct *H. polymorpha* AO into the secretory pathway, a hybrid gene was constructed encoding the leader sequence of the *S. cerevisiae* mating factor α (MFα) fused to *H. polymorpha* AO separated by a KEX2p cleavage site. This gene was placed under control of the inducible AO promoter (P<sub>AOX</sub>), and integrated into the genome of WT *H. polymorpha*. Strains were selected that contained multiple copies (>3 copies) of the expression cassette in the genome, as confirmed by Southern blotting (data not shown).

The resulting strain, WT::P<sub>AOX</sub>MFαAOX<sup>mc</sup>, grew normally on glucose, glycerol and methanol at rates identical to that of the WT host strain (data not shown). As expected, AO protein was not produced during growth of cells on glucose (data not shown). However, in Western blots prepared from cell extracts of methanol-grown cells, decorated with α-AO antibodies, two AO protein bands were observed (Fig. 1; lane 3). The lower protein band corresponds to WT AO protein, whereas the higher band corresponds to AO protein, which is glycosylated (Fig. 1; compare lanes 3 and 4) and, based on its molecular weight after endo H treatment, still contained the pror region of the MFα leader sequence.

AO protein and activity were never detected in the culture fluid of these cultures. This could be related to the AO production levels in cells grown in batch culture. Therefore, in order to maximize the production levels of recombinant AO protein, strain WT::P<sub>AOX</sub>MFαAOX<sup>mc</sup> was grown in a carbon-limited chemostat culture (D = 0.1 h<sup>−1</sup>) on a mixture of glucose and methanol. Electron microscopy revealed that cells grown under these conditions contained the expected numbers of peroxisomes (Van Dijken et al., 1976), a phenomenon paralleled by strong proliferation of ER-like vesicles (Fig. 2a). In immunocytochemical experiments using α-AO antibodies, labeling was observed on both the peroxisomal profiles and these vesicles (Fig. 2b). Subsequent cytochemical experiments unequivocally showed that AO enzymatic activity was strictly confined to the peroxisomal matrix (Fig. 2c). These results suggested that the MFαAO protein that accumulated in the ER-like vesicles had no enzymatic activity and therefore was most likely present as monomers. To seek further evidence for this, we aimed to determine the oligomeric state of the MFαAO protein as well as the FAD content. To avoid possible interference by endogenous WT AO with the recombinant AO in the purification procedures, we decided to use a strain in which the endogenous AOX gene was replaced by the MFαAO hybrid gene.

**Analysis of an *H. polymorpha* AO-deficient strain producing MFαAO fusion protein**

The *H. polymorpha* AOX::P<sub>AOX</sub>MFαAOX strain was not able to grow on methanol (Mut<sup>−</sup> phenotype; data not shown). In *H. polymorpha* Mut<sup>−</sup> cells, however, high P<sub>AOX</sub> induction levels can readily be achieved upon growth of cells in batch cultures on glycerol/methanol mixtures. In crude extracts, prepared from glycerol/methanol-grown AOX::P<sub>AOX</sub>MFαAOX cells, AO activity was not detectable; enzymatic activity was also not observed in the culture fluid of these cultures. Morphologically, strong proliferation of vesicles was observed. These structures represented the sole site of
AO protein in the cells, as was evident from immunocytochemistry (Fig. 3a).

These experiments also revealed that peroxisome biogenesis was not severely affected, except that the organelles present in the AOΔ::P_{AOX}MF_{AOX} cells were smaller than WT peroxisomes. The latter can be readily explained by the fact that a major protein constituent, AO protein, was absent from these organelles but had accumulated in the ER. These data also imply that import of hybrid AO into peroxisomes did not occur. Apparently, the artificial N-terminal secretion signal overrules the authentic peroxisomal sorting machinery.

Fig. 2. Overall morphology (a) of methanol-grown WT::P_{AOX}MF_{AOX} cells, showing the development of numerous vesicles. (b) Results of an immunocytochemical experiment using specific antibodies against AO. Labeling is located on peroxisomes, the nuclear membrane, and the vesicles, suggesting that the vesicles are of an ER nature. Immunocytochemically, AO activity is confined to peroxisomes (c). The proliferated ER vesicles (asterisks) show no reaction products, indicating the absence of AO activity. Protoplasts were incubated with CeCl₃ and methanol. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 0.5 µm.

Fig. 3. Immunocytochemical experiments, using α-AO antibodies on ultrathin sections of Δaox::P_{AOX}MF_{AOX} cells (a), grown on glycerol/methanol, showing the exclusive presence of AO protein in the vesicles. (b) A typical example of an identically grown WT Hansenula polymorpha cell in which AO protein is confined to peroxisomes. M, mitochondrion; N, nucleus; P, peroxisome. Arrows indicate ER-derived vesicles. Bar = 0.5 µm.
Characterization of MFzAO protein from AOM::P_{AOX}MFzAOX cells

Crude extracts, prepared from AOM::P_{AOX}MFzAOX and WT cells incubated in methanol media, were subjected to Western blot analysis using α-AO antibodies. These experiments revealed that the amount of AO protein present in AOM::P_{AOX}MFzAOX cells was reduced relative to the WT controls (Fig. 4).

To determine the oligomerization state and the FAD content of the vesicle-resident AO protein, crude extracts of glycerol/methanol-grown cells were subjected to sucrose gradient centrifugation. These data showed that AO from AOM::P_{AOX}MFzAOX cells was solely present at low density in the gradient, consistent with AO monomers (Fig. 5). The FAD content of these AO monomers was analyzed by fluorescence spectroscopy, which failed to resolve the presence of FAD. Taken together, these data indicate that the hybrid AO protein that had accumulated in the ER in AOM::P_{AOX}MFzAOX cells was exclusively present as monomers that had not bound FAD.

The finding that in AOM::P_{AOX}MFzAOX cells the AO levels are reduced compared to WT controls may be interpreted as meaning that the hybrid protein is less stable than its WT counterpart. To investigate this aspect in more detail, AOM::P_{AOX}MFzAOX cells were grown for 24 h in batch cultures on glycerol/methanol. Subsequently, excess glucose was added to the cultures, thus fully repressing the synthesis of AO protein. In WT cells, the addition of excess glucose leads to rapid degradation of the peroxisomes present in the cells via macroxenophagy, and thus to a rapid decrease in the level of AO protein (Dunn et al., 2005; Sakai et al., 2006). The fate of AO protein present in the AOM::P_{AOX}MFzAOX cells was followed by Western blot analysis. The data presented in Fig. 6 show that the levels of AO protein gradually decreased. As this protein is not subject to macroxenophagy, this suggests that the vesicle-resident AO is gradually degraded, possibly by the ER-associated degradation machinery (ERAD) (Werner et al., 1996). The reason for this degradation could be related to the fact that the ER quality control system recognizes the hybrid AO protein as being improperly folded, a known phenomenon for AO under conditions in which binding of FAD is prevented (Boteva et al., 1999).

Probably, the MFz signal sequence results in post-translational import of the hybrid AO protein into the ER (Brodsky et al., 1995), and thus the protein is expected to be completely synthesized in the cytosol prior to import into this compartment. This property allowed to investigate whether the hybrid AO protein was intrinsically competent to assemble into an active enzyme. This assumption is based on earlier observations that putative peroxisomal AO assembly factors are also functional in the cytosol, as exemplified by the presence of active oligomeric AO in the cytosol of peroxisome-deficient (pex) mutant cells that lack intact peroxisomes (Ozimek et al., 2005).

Therefore, we studied the fate of MFzAO in a pex3/ AOM::P_{AOX}MFzAOX double mutant. As expected, methanol-induced cells of this strain lacked peroxisomes (data not shown). In addition, in crude extracts prepared from such
cells, AO activity was invariably undetectable (data not shown). These results suggest that putative peroxisomal AO assembly factors, which are functional in the cytosol of pex mutants, were not able to perform their function in the cytosol of the double mutant cells.

In earlier studies, we demonstrated that the availability of FAD is of crucial importance for the correct assembly of AO (Evers et al., 1994, 1996). To investigate the possibility that limiting amounts of FAD could account for the default in MFzAO assembly, both the AOΔ::PaoxMFzAOX strain and the pex3/AOΔ::PaoxMFzAOX double mutant strain were grown on glycerol/methanol in the presence of 1 mM riboflavin. However, also under these conditions, AO activity could not be detected, consistent with the view that FAD is not the limiting factor in the AO assembly process in these cells (data not shown).

Analysis of strains producing an ISS–AO fusion protein

The results obtained indicated that the signal sequence of S. cerevisiae MFz efficiently sorted peroxisomal AO to the secretory pathway, although secretion was prohibited. We speculate that the inability to bind FAD was due to steric hindrance of the FAD-binding fold by the proregion of the leader sequence, as the FAD-binding site is near the N-terminus of the protein (Boteva et al., 1999). To test this possibility, we used an alternative sorting signal, namely the S. cerevisiae ISS. This signal differs from that of the MFz in that it consists only of a presequence, which is cleaved off upon import of the protein into the ER. As a result, the N-terminus of AO may be normally exposed under these conditions.

To obtain strains in which AO was sorted to the ER using the ISS, a hybrid gene was constructed encoding the ISS fused in-frame to the H. polymorpha AO gene. This gene was placed under the control of the inducible AOX promoter (Paox) and integrated at various copy numbers into the genome of WT H. polymorpha. The resulting strains grew on glucose at rates identical to the WT host strain (data not shown). In Western blots, prepared from crude extracts of strains with different copy numbers, shown). In Western blots, prepared from crude extracts of glucose at rates identical to the WT host strain (data not shown).

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To investigate the possibility that the extracellular AO is due to cell lysis, the WT::PaoxISSAOXmc strain was grown on methanol, and the appearance of AO activity in the culture fluid was analyzed. This revealed that extracellular AO activity appears already during the mid-exponential growth phase, which makes lysis as the cause of extracellular AO highly unlikely (data not shown). When vital staining of cells from the exponential cultures was performed, dead cells were not observed (data not shown).

Other evidence arguing against possible cell lysis was that two other major components of the peroxisomal matrix, dihydroxy acetone synthase (DHAS) and catalase (CAT), and the cytosolic protein elongation factor 1α (EF1α), were
clearly present in cell extracts but not detectable in the culture fluid by Western blot analysis (data not shown). Moreover, enzyme measurement revealed that the cytosolic enzyme formate dehydrogenase (FDH) was readily detectable in cell extracts but absent in the culture fluid (data not shown).

To ensure that the extracellular AO was indeed originating from the ISS–AO fusion, the secreted AO protein was N-terminally sequenced. Here, we made use of the fact that removal of the ISS upon import of the recombinant AO into the ER would result in a serine residue as the first amino acid. Sequencing of the extracellular AO indeed revealed that the first amino acid residue was a serine, thus confirming the recombinant origin of the extracellular AO protein.

The amount of AO protein detected in the culture fluid was limited relative to the total amount of AO produced (compare Figs. 7a and c). This suggests that bulk of the AO protein had accumulated intracellularly. Indeed, immunocytochemistry, using α-AO antibodies, on WT::PAOXISSAOXmc cells revealed that AO protein was present not only on the peroxisomal profiles but also on strongly proliferated ER-like structures (Figs. 8a and b). DHAS protein, however, was only present in peroxisomes, indicating that correct sorting of DHAS was unaffected in such cells (Fig. 8c).

To determine whether the hybrid AO protein was able to bind FAD, the recombinant protein was purified from an AOΔstrain producing the ISS–AO fusion protein. Immunocytochemistry revealed that, as in WT::PAOXISSAOX cells, strong proliferation of ER-like structures had occurred (Fig. 8d). These structures represented the sole sites of AO protein in the cells. Fluorescence analysis of the FAD content of recombinant AO, immunoprecipitated from crude extracts of AOΔ::PAOXMFzAOX, revealed that FAD was present in such precipitates, although at relatively low amounts (data not shown). From this, we concluded that a significant proportion of the ISS–AO fusion protein had bound FAD.

As already indicated, cells of strain WT::PAOXISSAOXmc are able to secrete active AO protein. However, the bulk of the recombinant protein remained intracellularly in a glycosylated form. Analysis of the AO protein sequence revealed that AO contains two putative N-glycosylation sites (Asn32

Fig. 8. Overall morphology (a) of methanol-grown WT::PAOXISSAOXmc cells, showing the presence of normal peroxisomes and the strongly proliferated ER-like structures. (b, c) Immunocytochemical experiments using α-AO (b) or α-DHAS (c) antibodies. AO labeling is located on peroxisomes and the ER-like structures. DHAS labeling was exclusively located on peroxisomal profiles, indicating that DHAS sorting was unaffected in these cells. (d) After labeling of Δaox::PAOXISSAOX cells using α-AO antibodies, AO protein is exclusively present on proliferated ER-like structures, but not on peroxisomes. N, nucleus; M, mitochondrion; P, peroxisome; V, vacuole. Arrows indicate ER-like structures. Bar = 0.5 μm.
and Asn155). On the basis of molecular weight estimation of the produced AO protein, we propose that one of these sites is glycosylated in vivo. We hypothesized that glycosylation of the recombinant AO protein may lead to a nonexportable protein conformation. This would explain the observation that the extracellular AO, which is active, is not glycosylated. To investigate this possibility, we investigated the effect of tunicamycin, which is a known inhibitor of glycosylation, on the secretion levels of AO protein. The *H. polymorpha* WT::PAOXISSAOX<sup>mc</sup> strain was grown on methanol for 12 h, when secretion of active AO had just started. Thereafter, tunicamycin was added to the culture. The results, shown in Fig. 9, indeed demonstrate that incubation of cells in the presence of tunicamycin leads to enhanced levels of extracellular AO protein.

**Discussion**

We have examined the fate of *H. polymorpha* AO upon artificial sorting of this peroxisomal matrix protein into the secretory pathway. WT *H. polymorpha* AO is an oligomeric flavoprotein that consists of eight identical subunits, each of which carries a flavin moiety (FAD) as the prosthetic group. AO is a typical example of a complex enzyme molecule that cannot spontaneously assemble *in vitro* from its constituent parts (recently reviewed by Ozimek *et al.*, 2005), and was used here as a model protein with which to investigate the requirements for the production and secretion of complex flavoproteins.

In methanol-grown *H. polymorpha*, AO is normally located in peroxisomes. To direct AO into the secretory pathway, we used the coding region of the leader sequence of *S. cerevisiae* MF<sub>a</sub> placed in front of the full-length homologous *H. polymorpha AOX* gene. The rationale of this approach was two-fold. First, the MF<sub>a</sub> signal functions well in *H. polymorpha*, as exemplified by the successful secretion of *A. niger* glucose oxidase (GOX), using this signal (Van der Heide *et al.*, 2002). It is relevant here that *A. niger* GOX is a dimeric flavoprotein that resembles AO in structure (Boteva *et al.*, 1999). Second, using the MF<sub>a</sub> leader sequence, the hybrid AO protein will most probably be post-translationally imported into the ER. This implies that the protein is completely synthesized in the cytosol prior to import into the ER, an order of events that largely resembles the normal import pathway of AO into peroxisomes.

Our data indicated that the MF<sub>a</sub>AO fusion protein is indeed sorted into the secretory pathway. Our results demonstrate that the hybrid AO protein remained in the secretory pathway and was not exported from the cell. Possibly, the conformation of the protein did not allow export. This hypothesis is consistent with the observation that the MF<sub>a</sub>AO fusion protein was exclusively present as a monomer that lacked FAD. The failure to bind FAD was not due to FAD limitation, as was evident from experiments performed under conditions of excess riboflavin. Monomeric AO, lacking FAD, is also highly unstable *in vitro* (De Hoop *et al.*, 1991), and readily aggregates, both *in vitro* and in intact cells. Thus, conformational changes of AO monomers related to the absence of FAD may cause the failure in secretion. Subsequently, the ER quality control system may recognize these proteins as being incorrectly folded, leading to the removal of these molecules by the ERAD machinery. This would be the most likely explanation for the observed gradual decrease of hybrid AO protein upon a shift of methanol-induced cells to conditions in which the AO protein is no longer synthesized.

The reason why MF<sub>a</sub>AO protein is not assembled is still not known with certainty. A likely explanation is based on the notion that the protein MF<sub>a</sub> presequence is not cleaved off, as suggested by the size of the protein relative to WT AO. The presence of this sequence could interfere (e.g. by steric hindrance or by affecting the structure) with the availability of the FAD-binding site, which is located at the N-terminus of the protein, and thus prevent binding of the cofactor. This would also be a plausible explanation for the finding that all MF<sub>a</sub>AO protein is directed to the ER. Gunkel *et al.* (2004) showed that Pex5p only binds AO when FAD is present. This possibility was strengthened by the data obtained using an alternative signal sequence to direct AO into the secretion pathway, namely the leader sequence of *S. cerevisiae* ISS. This signal differs from the MF<sub>a</sub> signal in that it is spliced off during import of the protein into the ER, with the advantage that the N-terminus of the ER-resident AO is now unaltered and normally exposed at these conditions.
ISS–AO was efficiently targeted to ER-like structures, despite the fact that the peroxisomal targeting signal (ARF) is still present at the C-terminus of the protein. This location of ISS–AO was accompanied by strong proliferation of these ER-like structures. Peroxisome biogenesis, however, was almost unaffected under these conditions.

In addition, active AO protein could be detected in the culture fluid of methanol-grown WT::P_AOXISS-AOX cells. The presence of extracellular AO was not due to cell lysis, as demonstrated by the fact that neither DHAS nor CAT was detectable in the growth medium. Furthermore, the activities of both EF1α and formate dehydrogenase (FDH), both cytosolic proteins, were also invariably absent in the culture fluid under these conditions. The extracellular AO protein is of recombinant origin, as became clear from N-terminal sequence analysis. Probably, AO secretion can be increased using an engineered AOX gene in which possible glycosylation sites are mutated.

In summary, we have shown that it is possible to redirect a complex peroxisomal flavoprotein, namely AO, to the secretion machinery and allow secretion of enzymatically active protein. Apparently, the secretory signal is able to overrule the peroxisomal targeting signal. This opens the way to develop methylotrophic yeasts as efficient cell factories for the production of this industrially important group of enzymes.

Acknowledgements

We thank Klaas Sjollema and Ineke Keizer for technical assistance in electron microscopy. M. van der Heide is supported by STW, I.J. van der Klei by an ALW/NWO Pionier-grant, and A.N. Leão by a grant from the State University of Rio de Janeiro (Brazil).

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