**Hansenula polymorpha** and *Saccharomyces cerevisiae* Pex5p’s recognize different, independent peroxisomal targeting signals in alcohol oxidase

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**Abstract** Peroxisomal alcohol oxidase (AO) from *Hansenula polymorpha* is inactive and partially mislocalized to the cytosol upon synthesis in *Saccharomyces cerevisiae*.

Co-production with *H. polymorpha* pyruvate carboxylase (HpPyc1p) resulted in AO activation, but did not import improve into peroxisomes.

We show that import of AO mediated by *S. cerevisiae* Pex5p is strictly dependent on the peroxisomal targeting signal 1 (PTS1) of AO and independent of HpPyc1p.

In contrast, HpPex5p-mediated sorting of AO into *S. cerevisiae* peroxisomes is independent of the PTS1, but requires an alternative PTS that is only formed when HpPyc1p is co-produced and most likely involves folding and co-factor binding to AO.

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**Keywords:** Peroxisome; Protein translocation; Pex5p; Enzyme activation; Yeast

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1. Introduction

Most peroxisomal matrix proteins contain a peroxisomal targeting signal 1 (PTS1) that consists of a tripeptide located at the extreme C-terminus of the protein. The PTS1-receptor, Pex5p, recognizes this signal via its C-terminal TPR (tetra-tricopeptide repeats) domain. The N-terminal half of Pex5p is important for binding to other components of the peroxisomal protein translocation machinery.

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The *S. cerevisiae* strains HpPEx5 and HpPYC1-HpPEx5, in which Sc *Pex5* was replaced by HpPEx5 under control of the glyceraldehyde-3-phosphate dehydrogenase promoter (*P<sub>GPD</sub>*) were constructed using PCR-directed integration [13]. The *S. cerevisiae HIS3* gene was amplified using primers “HIS start” and “HIS stop” (Table 2) and inserted into *Sma*I-digested *pBluescriptII KS(+),* resulting in *pBS-HIS3.* The *HIS3* gene was introduced downstream of *P<sub>GPD</sub>*, *HIS5* and the *CYC1* terminator (*T<sub>CYC1</sub>*) was recloned in *pBS-HIS3* digested with *SacI*/*DraI* (T4 DNA polymerase-blunt ended) fragment of *pGFP*-PEX5, containing the expression cassette with *P<sub>GPD</sub>*, *HIS5* and *CYC1* terminator (*T<sub>CYC1</sub>*). This plasmid, *pBS-HIS3*-PEX5*-Sc HIS3* was used as template to amplify the *Hp* fragment of *pHISP4*-PEX5 [15]. The *Sma*I and inserted fragment of 4730 bp was used to transform *S. cerevisiae* strains used in this study.

2.3. Miscellaneous

For production of green fluorescent protein (GFP)-SKL in *S. cerevisiae* WT and HpPEx5 strains, cells were transformed with *pgFP-SKL* [16].

2.4. Yarrowia lipolytica

Electron microscopy, immunocytochemistry, fluorescence microscopy [17], AO activity measurement and the separation of AO monomers and octamers [18] were performed as described. SDS-PAGE and Western blotting were performed by established techniques.

### Table 1

*S. cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>S. cerevisiae</em> gene</th>
<th>H. polymorpha gene</th>
<th>Source</th>
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<tr>
<td><em>Pex5</em> (UTL7A <em>pex5</em>)</td>
<td>+</td>
<td>+</td>
<td>[9]</td>
</tr>
<tr>
<td>WT (CEN.PK2)</td>
<td>+</td>
<td>+</td>
<td>Dr. Peter Koetter</td>
</tr>
<tr>
<td><em>pvc1, pvc2</em> (CEN.PK700)</td>
<td>–</td>
<td>+</td>
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<tr>
<td>HpPYC1</td>
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<td>This study</td>
</tr>
<tr>
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<td>+</td>
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<tr>
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<td>–</td>
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<td>This study</td>
</tr>
<tr>
<td>HpPYC1-HpPEx5-AO&lt;sup&gt;mut&lt;/sup&gt;</td>
<td>–</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
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<td>–</td>
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<tr>
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### Table 2

Oligonucleotide primers used in this study

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<td>AOX ATG</td>
<td>GAGCTAGCTAAAAATGGCCATTCGAGG</td>
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<tr>
<td>AOX stop</td>
<td>GCTGGAGGCTCTTGGCGCTACAT</td>
</tr>
<tr>
<td>HIS start</td>
<td>GCGGAATTCCTAGCATGATCAG</td>
</tr>
<tr>
<td>HIS stop</td>
<td>GTTAGGCGGACCTATGACGCAAC</td>
</tr>
<tr>
<td><em>P&lt;sub&gt;GPD&lt;/sub&gt;</em>-<em>HpPEx5</em> start</td>
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</tr>
<tr>
<td><em>P&lt;sub&gt;GPD&lt;/sub&gt;</em>-<em>HpPEx5</em> stop</td>
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<tr>
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<td>HpPEx5 colony PCR 2</td>
<td>GTGGTTGTTGATTGATCAG</td>
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<tr>
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<td><em>P&lt;sub&gt;GPD&lt;/sub&gt;</em>-<em>HpPEx5</em> start</td>
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</tr>
<tr>
<td><em>P&lt;sub&gt;GPD&lt;/sub&gt;</em>-<em>HpPEx5</em> stop</td>
<td>CTATGGAACATCCCTGGGACATCAG</td>
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<tr>
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</tr>
<tr>
<td>HpPEx5 colony PCR 2</td>
<td>GTGGTTGTTGATTGATCAG</td>
</tr>
</tbody>
</table>

<sup>a</sup>Underlined nucleotides indicate restriction sites.

### 3. Results

#### 3.1. *H. polymorpha* AOX is activated in *S. cerevisiae* expressing *HpPYC1*

To test whether HpPyc1p activates AO in *S. cerevisiae*, a strain was constructed that contained the *H. polymorpha* *AOX* and *PYC1* genes, but lacked *ScPYC1* and *ScPYC2* (strain HpPYC1-AO, see Table 1). In these cells HpPyc1p levels were obtained that were comparable to those present in methanol-grown *H. polymorpha* WT cells, however the AO protein levels were much lower in HpPYC1-AO relative to *H. polymorpha* (*Hp*, Fig. 1A).

AO enzyme activities of 0.2 (±0.02) U/mg protein were measured in cell extracts of HpPYC1-AO. As expected AO activity was not detectable in the control *S. cerevisiae* strain that produces AO, but lacks HpPyc1p (WT-AO). These data indicate that co-expression of *HpPYC1* with *HpAOX* is sufficient to obtain enzymatically active AO in *S. cerevisiae*. Like the AO protein levels also the enzyme activities were significantly lower in *S. cerevisiae* HpPYC1-AO compared to methanol-grown *H. polymorpha* WT controls (*Hp*, Fig. 1A [4]).

Sucrose density centrifugation of cell free extracts confirmed that in the presence of HpPyc1p AO octamers were formed in *S. cerevisiae*, whereas in cells lacking HpPyc1p AO remained monomeric (*Fig. 1B*). Upon cytochemical staining of AO enzyme activity, AO activity was only detected inside peroxisomes in cells producing both AO and HpPyc1p (data not shown).
3.2. Co-expression of H. polymorpha PYC1 or PEX5 does not improve the efficiency of AO sorting in S. cerevisiae

AO localization studies using WT-AO cells (Fig. 2) revealed that only a minor portion of the AO protein was present in peroxisomes, whereas the bulk was mislocalized to the cytosol and nucleus, where it often was present in protein aggregates. This dual localization was unchanged in HpPYC1-AO cells (data not shown), which indicates that co-expression of HpPYC1 does not significantly improve AO import into S. cerevisiae peroxisomes.

Recently, we showed that AO contains an alternative, yet unknown PTS, that most likely is formed upon HpPyc1p-mediated FAD binding and recognized by the N-terminal domain of HpPex5p [3]. We next tested whether replacement of ScPEX5 by HpPEX5 improved AO import. In a strain producing both HpPyc1p and HpPex5p (HpPYC1-HpPEX5-AO) only a minor portion of AO was imported into peroxisomes (Fig. 2D). This low import efficiency was not due to limiting HpPex5p levels, because these were similar as in H. polymorpha WT controls (Fig. 3A).

3.3. HpPex5p and ScPex5p recognize different peroxisomal targeting signals in AO

In a strain in which ScPEX5 was replaced by HpPEX5 without co-expression of HpPYC1, anti-AO specific labelling was absent in peroxisomes (Fig. 2C), which confirms our earlier data that HpPex5p does not bind AO via its PTS1 [4]. However, ScPex5p most likely imports AO via its PTS1, because AO is partially imported into peroxisomes of WT-AO cells (Fig. 2A). To test this, the PTS1 (–ARF) of AO was destroyed by changing it into –ARA (AOmut) [3]. In cells of this strain (WT-AO mut) sorting of AO to peroxisomes was fully abolished (Fig. 2B). However, AOmut was imported into peroxisomes in cells producing both HpPyc1p and HpPex5p (HpPYC1-HpPEX5-AO mut; Fig. 2E).
Hence, in *S. cerevisiae* the alternative AO PTS is formed in an HpPyc1p-dependent way and recognized by HpPex5p. However, because no AO import was observed in HpPYC1-AO<sup>com</sup> cells (data not shown), we conclude that ScPex5p is unable to recognize this alternative PTS.

All *S. cerevisiae* strains that produced AO in the presence HpPyc1p showed similar AO enzyme and protein levels, independent of the localization of the protein. This indicates that activation of AO in *S. cerevisiae* is not dependent on import of the protein into the peroxisomal matrix (Fig. 3B).

### 3.4. HpPex5p is not fully functional in PTS1 protein import in *S. cerevisiae*

Partial import of AO in strains producing both HpPex5p and HpPyc1p suggests that HpPex5p is not fully functional in the heterologous host. We therefore analysed import of green fluorescent protein containing a PTS1 in cells expressing *Hp*PEX5 (HpPEX5-GFP-SKL). As shown in Fig. 4, most GFP fluorescence was mislocalized to the cytosol, whereas only part of the fluorescence was found in punctuated structures. In contrast, GFP-fluorescence was confined to punctuated structures in WT controls. The growth rate and final yield of HpPEX5-GFP-SKL cells on oleic acid media was reduced as compared to WT controls (data not shown), which is also indicative for a partial matrix protein import defect.

### 4. Discussion

Here, we show that pyruvate carboxylase of *H. polymorpha* (HpPyc1p) is sufficient to activate AO in the heterologous host *S. cerevisiae*. We previously cloned *HpPYC1* by complementation of a *H. polymorpha* mutant defective in AO assembly [4,19]. This finding was unexpected since HpPyc1p is an enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate. Mutational analysis revealed that not the enzyme activity, but another function of this protein fulfills a role in AO activation. Most likely HpPyc1p mediates FAD-binding via a yet unknown mechanism.

*S. cerevisiae* contains two pyruvate carboxylase genes, *PYC1* and *PYC2*. While the corresponding proteins are approximately 75% identical to HpPyc1p, they are unable to activate AO.

Our data suggest that HpPyc1p is the only *H. polymorpha* protein that is specifically required for AO activation. All *S. cerevisiae* strains that produced AO in the presence HpPyc1p showed similar AO activity levels indicating that activation is not dependent on import into peroxisomes.

Our data reveal that ScPex5p binds the PTS1 of AO (–ARF). This PTS1 is a conserved variant of the classical PTS1, –SKL, which most likely has a relatively low affinity for ScPex5p [20,21]. This may explain the low efficiency of ScPex5p mediated AO import. Replacement of *ScPEX5* by *HpPEX5* did not improve import, because HpPex5p is not fully functional in *S. cerevisiae* (Fig. 4). Other examples have been reported where the PEX5 gene from one organism fails to fully complement PEX5 deletion in another organism (e.g., *Pichia pastoris* PEX5 and *Penicillium chrysogenum* PEX5 do not fully restore the PTS1 import defect in *H. polymorpha* pex5 cells [15,22]).

HpPex5p binds a yet unknown, alternative PTS in AO, which is not recognized by ScPex5p. On the other hand, HpPex5p apparently is unable to recognize the AO PTS1 (–ARF) in the context of the AO protein [3]. However, fusion of this PTS1 to GFP (GFP-ARF) results in import of this reporter protein into *H. polymorpha* peroxisomes [2]. A likely explanation is that residues that lie upstream from the C-terminal –ARF interfere with AO-binding to the PTS1-binding TPR domain of HpPex5p, but not of ScPex5p.

Because ScPex5p-mediated AO targeting does not depend on HpPyc1p, it is possible that both FAD-containing and FAD-lacking AO is imported into peroxisomes by ScPex5p. The special AO PTS probably prevents import of FAD-lacking AO in *H. polymorpha*. Our results imply that HpPex5p is capable to sense binding of FAD to the cargo protein. *S. cerevisiae* may have developed a similar mechanism for import of the peroxisomal flavo-enzyme acyl CoA-oxidase, which is also imported via the N-terminal domain of ScPex5p [16,23]. The involved functional domains in the N-terminus of HpPex5p and ScPex5p are not the same, because ScPex5p does not recognize the alternative PTS of AO, whereas the region in ScPex5p that is essential for binding of acyl CoA oxidase [23] is absent in HpPex5p.

It is possible that co-factor binding in the cytosol is a very common feature in peroxisomal protein translocation, like in the Tat protein translocation pathway. For instance, in *Y. lipolytica* proper assembly of the heteropentameric acyl-CoA oxidase is essential to allow import into peroxisomes [24]. The importance of co-factor binding and folding in peroxisomal protein import may so far have been overlooked due to the common use of non-peroxisomal reporter proteins in peroxisomal protein translocation studies.

If the N-terminal domains of Pex5p’s can sense co-factor binding, it is tempting to speculate that in PTS2 protein import the Pex7p accessory proteins (Pex18p, Pex21p, Pex20p and the long form of human Pex5p) fulfil a similar role.

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Fig. 4. Fluorescence microscopy of WT-GFP-SKL (A,B) and HpPEX5-GFP-SKL cells (C,D). A,C – bright field; B,D – fluorescence images.
References


