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The transcarboxylase domain of pyruvate carboxylase is essential for assembly of the peroxisomal flavoenzyme alcohol oxidase

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Keywords
pyruvate carboxylase; peroxisome; Hansenula polymorpha; alcohol oxidase; transposon mutagenesis; FAD.

Abstract
Pyruvate carboxylase (Pyc1p) has multiple functions in methylotrophic yeast species. Besides its function as an enzyme, Pyc1p is required for assembly of peroxisomal alcohol oxidase (AO). Hence, Pyc1p-deficient cells share aspartate auxotrophy (Asp^−) with a defect in growth on methanol as sole carbon source (Mut^−). To identify regions in Hansenula polymorpha Pyc1p that are required for the function of HpPyc1p in AO assembly, a series of random mutations was generated in the HpPYC1 gene by transposon mutagenesis. Upon introduction of 18 mutant genes into the H. polymorpha PYC1 deletion strain (pyc1), four different phenotypes were obtained, namely Asp^− Mut^−, Asp^− Mut^+, Asp^+ Mut^−, and Asp^+ Mut^+. One mutant showed an Asp^+ Mut^− phenotype. This mutant produced HpPyc1p containing a pentapeptide insertion in the region that links the conserved N-terminal biotin carboxylation domain (BC) with the central transcarboxylation (TC) domain. Three mutants that were Asp^− Mut^− contained insertions in the TC domain, suggesting that this domain is important for both functions of Pyc1p. Analysis of a series of constructed C-terminal and N-terminal truncated versions of HpPyc1p showed that the TC domain of Pyc1p, including the region linking this domain to the BC domain, is essential for AO assembly.

Introduction
Peroxisomal matrix proteins are synthesized in the cytosol and posttranslationally imported into peroxisomes. Correct sorting requires peroxisomal targeting signals (PTS) that are recognized by one of the two known PTS receptor proteins, Pex5p or Pex7p. These receptors are soluble proteins that function as cycling receptors and may enter the peroxisomal matrix in complex with their cargo. For a recent review, see Heiland & Erdmann (2005).

Most peroxisomal matrix proteins contain a PTS1 sequence, which consists of three amino acids (SKL or conserved variants thereof), that is located at the extreme C-terminus (Heiland & Erdmann, 2005). The PTS1 is recognized by the C-terminal tetratricopeptide (TPR) repeat domain of Pex5p (Gatto et al., 2000). The N-terminal part of Pex5p is involved in binding to the peroxisomal membrane (docking), the translocation process, and recycling to the cytosol (Otera et al., 2002; Costa-Rodrigues et al., 2004).

Interestingly, several examples exist of peroxisomal matrix proteins that are sorted to peroxisomes in a Pex5p-dependent way, but which either lack a PTS1 or contain a redundant PTS1. An example is Saccharomyces cerevisiae acyl-CoA oxidase. This peroxisomal flavoenzyme does not have a PTS1, but its sorting is dependent on ScPex5p. The association of acyl-CoA oxidase with ScPex5p does not require the C-terminal PTS1-binding domain of Pex5p, but a region in the ScPex5p N-terminus (Klein et al., 2002; Schafer et al., 2004). Alcohol oxidase (AO) of Hansenula polymorpha is an example of a peroxisomal protein that does contain a functional PTS1, which, however, is redundant for targeting of the protein to peroxisomes. Like acyl-CoA oxidase, AO is a flavoenzyme. We previously showed that import of AO can be mediated by a truncated Pex5p protein, which lacks the C-terminal TPR domain (Gunkel et al., 2003), a phenomenon that has also been described for bakers’ yeast acyl-CoA oxidase (Schafer et al., 2004). Our previous data suggest that AO is only recognized by Pex5p after binding of its cofactor FAD (Evers et al., 1994, 1996; Gunkel et al., 2003). Hence, FAD binding may result in the formation of an as yet unknown PTS in AO (Gunkel et al., 2003).

In H. polymorpha mutants defective in the cytosolic protein pyruvate carboxylase (HpPyc1p), newly synthesized AO monomers fail to bind FAD and, as a consequence, are...
not sorted to peroxisomes or assembled into enzymatically active octamers (Ozimek et al., 2003). The same phenomenon was observed in the related yeast *Pichia pastoris* (Ozimek et al., 2003). HpPyc1p is an anaplerotic enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate generated from pyruvate. The function of HpPyc1p in AO assembly does not require enzymatically active HpPyc1p, but involves a novel, additional function of the protein (Ozimek et al., 2003). The fate of newly synthesized AO protein in *H. polymorpha* cells deleted for *PYC1* is similar to that observed in cells blocked in synthesis of the FAD (Evers et al., 1994), suggesting that HpPyc1p plays a role in binding FAD to AO monomers. In line with this assumption is the observation that HpPyc1p specifically interacts with AO (Ozimek et al., 2003).

How would HpPyc1p function in the FAD-binding process? One possibility is that HpPyc1p interacts with newly synthesized AO monomers to mediate or stabilize a protein conformation that is competent for FAD binding. Another possibility is that HpPyc1p is actively involved in donating FAD to AO, which implies that HpPyc1p (transiently) binds FAD.

In order to elucidate the molecular mechanisms involved in the novel function of HpPyc1p, we aimed to delineate the region(s) in HpPyc1p required to activate AO. An NCBI Conserved Domain Search (Marchler-Bauer et al., 2005) did not reveal any unique domain in HpPyc1p that is absent in Pyc proteins of nonmethylotrophic yeast species. Instead, like all other known eukaryotic Pyc proteins, HpPyc1p contains three functional domains, namely an N-terminal biotin carboxylation (BC) domain, a central transcarboxylation (TC) domain, and a C-terminal biotin carboxyl carrier (BCC) domain (Attwood, 1995). The BC domain is involved in ATP-dependent carboxylation of biotin. The TC domain transfers a carboxyl group from biotin to pyruvate, whereas the BCC domain binds biotin (Attwood & Wallace, 2002). None of these domains contains an FAD-binding motif. However, the BC domain contains an ATP-binding site.

Using different mutagenesis approaches, we dissected the region in HpPyc1p required for AO assembly. The outcome of these studies is presented in this article.

**Materials and methods**

**Organisms and growth conditions**

The *H. polymorpha* strains used in this study were wild-typeNCYC 495 leu1.1 (Gleeson & Sudbery, 1988), pyc1 leu1.1 (Ozimek et al., 2003), and ass3–110 (van Dijk et al., 2002). Cells were cultivated at 37 °C in mineral media (Van Dijken et al., 1976), supplemented with 0.5% glucose, 0.5% methanol, or a mixture of 0.1% glycerol and 0.5% methanol as carbon sources. As nitrogen source, 0.2% ammonium sulfate or 0.25% methanol was used. Solid media contained 0.67% Yeast Nitrogen Base without amino acids (Difco, Sparks, MD), and were supplemented with 1% glucose (YN) or 0.5% methanol (YNM) and 2% agar. When needed, aspartate was added to a concentration of 60 mg L⁻¹.

*Escherichia coli* DH5α was used for cloning purposes, and cultivated as described (Sambrook et al., 1989).

**Genetic manipulations and yeast transformation**

All standard DNA procedures were performed as previously described (Sambrook et al., 1989). Electrottransformation of *H. polymorpha* cells was carried out as detailed by Faber et al. (1994). The HpPYC1 gene of original mutant ass3–110 (Ozimek et al., 2003) was sequenced after amplification by PCR using chromosomal DNA isolated from ass3–110 cells, and primers PYC-ATG and PYC-STOP (Table 1).

**Transposon mutagenesis of H. polymorpha PYC1**

The *H. polymorpha* PYC1 gene was cloned from pHIPX5-PYC1 (Ozimek et al., 2003) as an NcoI (Klenow filled-in)/SphI fragment into pHIPX6 (Kiel et al., 1995) digested with BamHI (Klenow filled-in) and SphI. The resulting plasmid, pHIPX6-PYC1, was subjected to NotI digestion followed by Klenow fill-in and religation to eliminate the NotI restriction site. The final construct was mutagenized with the transposon-based MGS mutation generation system (Finnzymes OY, Espoo, Finland). Plasmids were used to transform *E. coli*, and colonies with transposon-containing plasmids were selected on the basis of chloramphenicol resistance gene present in the transposon.

Further restriction analysis of the resulting plasmids with BamHI/HindIII allowed selection of mutants that carried transposons in the HpPYC1 gene, but not in the vector backbone. The insertion sites were sequenced using the

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
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</tr>
<tr>
<td>CAM reverse</td>
<td>GGGATATCATCGAAATCCAAAGAAACGACG</td>
</tr>
<tr>
<td>PYC1 trunc. 1</td>
<td>GGGGATATCATCAGAAGACTGAGTGGATGACATCC</td>
</tr>
<tr>
<td>PYC1 trunc. 2</td>
<td>GGGGATATCATCAGAAGACTGAGTGGATGACATCC</td>
</tr>
<tr>
<td>PYC1 trunc. 3</td>
<td>CCGATCAGCCAGACGAGGAGC</td>
</tr>
<tr>
<td>PYC1-STOP</td>
<td>CTTCCATGGCCAGGTGAGG</td>
</tr>
<tr>
<td>PYC-ATG</td>
<td>ATAGGGCGAATTGGGGATCGATC</td>
</tr>
<tr>
<td>SAK001</td>
<td>GCGGCCGCGGTGTTAACCATTACGGCAAGCCCTAATTTGG</td>
</tr>
<tr>
<td>SAK002</td>
<td>GGGCAGCTTATGTTGTGGGTGTTGGTGAATGGCCCATACGG</td>
</tr>
<tr>
<td>SAK011</td>
<td>CCATCACCCTTGTATGAGC</td>
</tr>
<tr>
<td>SAK014</td>
<td>GGAAGCGCGCGCGCATGCCCAGGGATGAGACATCC</td>
</tr>
<tr>
<td>SAK015</td>
<td>GGGGATATCATGCAATCCAAGAACAGAGC</td>
</tr>
</tbody>
</table>

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The 2.5-kb fragment coding for amino acids 1–815 of HpPyc1p was cloned into a 7.4-kb BamHI (Klenow filled-in)/SphI fragment of pHIPX4-HNBESX (K.B. Rechinger, unpublished results). The 2.5-kb fragment coding for amino acids 1–815 of HpPyc1p was generated by PCR using primers SAK014 and SAK015, and cloned as a NotI-digested insert into the 7.9-kb NotI/SmaI-digested vector pSAK1. The plasmid was designated pSAK1-PYC1\(^{1–815}\), and contained the truncated gene under control of the strong \(P_{AOX}\) promoter.

A fragment coding for amino acids 480–1061 of HpPyc1p containing a C-terminal His\(_6\)-tag (under control of \(P_{AMO}\)) was obtained by PCR using primers PYC1 trunc.1 and SAK011. The 1.8-kb product was digested with Sphi and ligated into a 7.4-kb BamHI (Klenow filled-in)/SphI fragment of pSAK4. The resulting plasmid was designated pSAK4-PYC1\(^{480–1061}\)–His\(_6\). Vector pSAK4 was cloned by ligation of a 1.8-kb Asp718I/NheI fragment of pSAK1 into the 7.3-kb fragment of pHIPX5-stuffer (M. Komori, unpublished results), digested with the same enzymes.

The plasmids were sequenced (Baseclear, Leiden, the Netherlands) and used to transform \(H.\ \textit{polymorpha}\ pyc1\) leu1.1 cells, yielding strains \(PYC1\)\(^{480–1061}\), \(PYC1\)\(^{512–1175}\), \(PYC1\)\(^{561–1175}\), \(PYC1\)\(^{1–560}\), and \(PYC1\)\(^{1–815}\), respectively.

### Isolation of His\(_6\)-tagged versions of HpPyc1p and HpPyc1\(^{13}\)p

Construction of the plasmid pQE60-PYC1 for the expression of a C-terminally His\(_6\)-tagged HpPyc1p in \(E.\ coli\) has been described previously (Ozimek et al., 2003). To obtain the analogous plasmid for expression of the His\(_6\)-tagged version of HpPyc1\(^{13}\)p, a BamHI/PstI fragment of the HPYC1 gene (963 bp) in pQE60-PYC1 was replaced by the fragment of HPYC1\(^{13}\) digested with the same enzymes (978 bp). The resulting plasmid pQE60-PYC1\(^{13}\) was used to transform \(E.\ coli\) SG13009(pREP4). Purification of both

---

### Table 2. \(H.\ \textit{polymorpha}\) and \(E.\ coli\) strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>(H.\ \textit{polymorpha}\ NCYC 495 leu1.1</td>
<td>Gleson &amp; Sudbery (1988)</td>
</tr>
<tr>
<td>pyc1</td>
<td>(H.\ \textit{polymorpha}\ NCYC 495 pyc1 leu1.1</td>
<td>Ozimek &amp; Sudbery (1995)</td>
</tr>
<tr>
<td>PYC1(^{1–560})</td>
<td>pyc1 containing plasmid pHIPX6-PYC1</td>
<td>This study</td>
</tr>
<tr>
<td>PYC1(^{1–20})</td>
<td>pyc1 containing plasmid pHIPX6-PYC1(^{1–20})</td>
<td>This study</td>
</tr>
<tr>
<td>PYC1(^{456–561})</td>
<td>(H.\ \textit{polymorpha}\ NCYC 495 asp3–110 leu1.1, Initial Mutant</td>
<td>van Dijk et al. (2002)</td>
</tr>
<tr>
<td>PYC1(^{1–560})</td>
<td>pyc1 containing plasmid pSAK1-PYC1(^{1–560})</td>
<td>This study</td>
</tr>
<tr>
<td>PYC1(^{480–1175})</td>
<td>pyc1 containing plasmid pSAK1-PYC1(^{480–1175})</td>
<td>This study</td>
</tr>
<tr>
<td>PYC1(^{1–815})</td>
<td>pyc1 containing plasmid pHIPX6-PYC1(^{1–815})</td>
<td>This study</td>
</tr>
<tr>
<td>PYC1(^{512–1175})</td>
<td>pyc1 containing plasmid pHIPX6-PYC1(^{512–1175})</td>
<td>This study</td>
</tr>
<tr>
<td>PYC1(^{561–1175})</td>
<td>pyc1 containing plasmid pHIPX6-PYC1(^{561–1175})</td>
<td>This study</td>
</tr>
<tr>
<td>pyc1-CAM</td>
<td>pyc1 with a one-copy integration of plasmid pHIPX5-PYC1</td>
<td>Ozimek et al. (2003)</td>
</tr>
<tr>
<td>E. coli SG13009(pREP4)</td>
<td>pyc1 containing plasmid pQE60-PYC1</td>
<td>Ozimek et al. (2003)</td>
</tr>
<tr>
<td>E. coli SG13009(pREP4)</td>
<td>pyc1 containing plasmid pQE60-PYC1(^{13})</td>
<td>This study</td>
</tr>
</tbody>
</table>

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proteins was performed as previously described (Ozimek et al., 2003).

**AO/HpPyc\(^{13}\p\) binding studies**

Sepharose beads containing covalently bound AO or bovine serum albumin (BSA) were prepared as described by Evers et al. (1993). Binding studies using purified HpPyc1\(^{13}\p\) as well as HpPyc1p were performed as described previously (Ozimek et al., 2003).

**Fluorescence measurements**

Fluorescence spectra were obtained using a Fluorolog 3.2.2 (Horiba Jobin Yvon) spectrofluorometer equipped with a thermostatically controlled cuvette holder. All measurements were performed at room temperature (22 °C) using purified His\(_6\)-tagged HpPyc1p and HpPyc1\(^{13}\p\) in 25 mM Tris-HCl (pH 7.0) supplemented with 50 mM KCl and 1 mM dithiothreitol. The absorbance of the samples at 280 nm was kept below 0.1 to minimize the inner filter effect. Excitation and emission slits were kept at 2 nm. The spectrum of a blank solution, containing all components except the protein, was subtracted from each sample spectrum. All collected spectra were corrected for wavelength-dependent instrumental response characteristics.

**Circular dichroism measurements**

Circular dichroism (CD) measurements were performed using using a Jasco J-715 spectropolarimeter, equipped with a Peltier temperature control system set at 20 °C. Circular dichroism measurements were performed at room temperature (22 °C). Circular dichroism measurements were performed at room temperature (22 °C). The plasmids were introduced into the H. polymorpha PYC1 gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the HpPyc1p gene, but not in the vector backbone.

**Biochemical methods**

AO activity was measured as described by Verduyn et al. (1984) in crude extracts, prepared according to van der Klei et al. (1991). AO monomers and octamers were separated by velocity centrifugation in sucrose density gradients (Good-
However, one transformant (strain PYC113) was obtained that was Asp\(^+\)/Mut\(^{-}\), which points to a specific defect in AO assembly.

These data demonstrate that the enzymatic function of HpPyc1p and its function in AO assembly can be separated.

**Analysis of strains PYC1\(^3\), PYC1\(^{14}\), PYC1\(^{15}\) and PYC1\(^{16}\)**

Four transformants (strains PYC1\(^3\), PYC1\(^{14}\), PYC1\(^{15}\) and PYC1\(^{16}\); Fig. 2 and Table 3) showed an Asp\(^-\)/Mut\(^-\) phenotype, suggesting that the regions altered in the HpPyc1p proteins in these strains are important for both HpPyc1p enzyme activity and its function in AO assembly. In PYC1\(^3\), the pentapeptide insertion occurred at the N-terminus. The other three (PYC1\(^{14}\), PYC1\(^{15}\) and PYC1\(^{16}\)) all contained the insertion in the TC domain.

To test whether the insertions that occurred in these strains affected the HpPyc1p protein levels, the HpPyc1p levels in these strains were analyzed by Western blotting using anti-HpPyc1p antibodies. As shown in Fig. 3, each of the Asp\(^-\)/Mut\(^-\) mutant strains produced HpPyc1p protein, although the levels were reduced relative to the control strain producing wild-type HpPyc1p. The lowest amounts of HpPyc1p were detected in strains PYC13 and PYC114.

Enzyme activity measurements revealed that neither of these strains showed AO activities that were higher than those observed in \(pyc1\) control cells [ranging from 0.02 to 0.08 U mg\(^{-1}\) protein (Ozimek et al., 2003); Fig. 3].

Using a strain containing the HpPYC1 gene under control of the P\(_{AMO}\) promoter (\(H. polymorpha pyc1::P_{AMO}PYC1\)), we previously showed that strongly reduced HpPyc1p levels are sufficient to activate AO (Ozimek et al., 2003). We cultivated cells of this strain at conditions that resulted in comparable HpPyc1p levels as observed in the four Asp\(^-\)/Mut\(^-\) strains. At these levels of HpPyc1\(^{1WT}\)p, AO activities of 0.41 U mg\(^{-1}\) were observed, indicating that the amount of HpPyc1p was

---

**Table 3. Mutant HpPyc1p proteins obtained by pentapeptide insertions**

<table>
<thead>
<tr>
<th>Mutant phenotype</th>
<th>Mutant number</th>
<th>Position and sequence of the pentapeptide insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp(^+), Mut(^-)</td>
<td>1, 2</td>
<td>21 LRPHL</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>52 IAAAA</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>123 CGRNS</td>
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<tr>
<td></td>
<td>6</td>
<td>124 CGRSG</td>
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<tr>
<td></td>
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<td>139 VRPHG</td>
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<tr>
<td></td>
<td>9</td>
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<td>10</td>
<td>353 CRRMQ</td>
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<td></td>
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<td>1106 MRRHP</td>
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<tr>
<td></td>
<td>20</td>
<td>1163 SAAAD</td>
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<tr>
<td>Asp(^-)/Mut(^-)</td>
<td>17</td>
<td>901 CGRIK</td>
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<tr>
<td></td>
<td>18</td>
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<td>754 SAAAI</td>
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<tr>
<td></td>
<td>16</td>
<td>794 CGRNC</td>
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</table>

Sequences of the pentapeptide insertions obtained by transposon mutagenesis with the corresponding phenotype of \(pyc1\) cells producing the different mutant HpPyc1p proteins. The phenotypes were determined based on the appearance of colonies on solid media relative to \(pyc1\) cells harboring the empty vector (\(pyc1\)) and \(pyc1\) cells expressing HpPYC1 from pHIPX6-PYC1 (PYC1\(^{1WT}\)). Mut\(^-\) and Asp\(^-\) strains grew like PYC1\(^{1WT}\) controls on media containing methanol/aspartate or glucose without aspartate, respectively (checked after 2 days of incubation). Strains were classified Mut\(^-\) or Asp\(^-\) when no colonies had appeared after 7 days of incubation on media containing methanol/aspartate or glucose without aspartate, respectively. Strains were scored as Asp\(^-\)/Mut\(^-\) when, on glucose media lacking aspartate, no colonies were observed after 2 days of incubation, but had appeared after 4 days of incubation.

---

**Fig. 1.** Complementation of \(Hansenula polymorpha\) \(pyc1\) cells by pHIPX6-PYC1. HpPYC1 expressed from plasmid pHIPX6, under control of the PEX3 promoter, complements the Mut\(^-\)/Asp\(^-\) phenotype of \(Hansenula polymorpha\) \(pyc1\) cells. Cells were grown in liquid media containing glucose without aspartate (a) or methanol plus aspartate (b). WT, wild-type cells containing the empty vector pHIPX6; \(pyc1\), \(pyc1\) cells containing the empty vector; PYC1\(^{1WT}\), \(pyc1\) strain expressing wild-type HpPYC1 from pHIPX6-PYC1. Growth is expressed as OD at 660 nm (\(OD_{660\,nm}\)).
sufficient for AO assembly (Fig. 3). In cells of the PYC115 and PYC116 strains, the HpPyc1p levels were at least as high as in the pyc1::PAMOPYC1 cells. On the basis of these data, we conclude that the defect in AO assembly in PYC115 and PYC116 is not due to limiting HpPyc1p levels.

PYC113 is specifically affected in AO assembly

The phenotype of mutant PYC113 (Asp+ Mut+) suggests a specific defect in AO assembly. This was supported by detailed growth experiments (Fig. 4), which revealed that PYC113 cells grew on glucose-containing media supplemented with aspartate. Cell extracts were analyzed by Western blotting using antibodies against HpPyc1p. The HpPyc1p levels in cells of the mutant strains were compared with that in cells of a strain producing artificially reduced levels of wild-type HpPyc1p (PAMOPYC1), which were grown on glycerol/methanol/aspertate media in the presence of ammonium sulfate to repress PAMO. The artificially reduced levels of wild-type HpPyc1p still resulted in AO assembly (0.41 vs. 0.02 U mg\(^{-1}\) protein observed in pyc1 control cells). The AO enzyme activities in cells of strains PYC13, PYC114, PYC115 and PYC116 were in the same range as observed in the pyc1 control cells. For the Western blot, equal amounts of protein were loaded per lane. To visualize the low HpPyc1p levels in cells of the mutant strains, relatively long exposure times were used.

In cells of the PYC115 and PYC116 strains, the HpPyc1p levels were at least as high as in the pyc1::PAMOPYC1 cells. On the basis of these data, we conclude that the defect in AO assembly in PYC115 and PYC116 is not due to limiting HpPyc1p levels.

**PYC113 is specifically affected in AO assembly**

The phenotype of mutant PYC113 (Asp+ Mut+) suggests a specific defect in AO assembly. This was supported by detailed growth experiments (Fig. 4), which revealed that PYC113 cells grew on glucose-containing media that lacked aspartate, as did the wild-type control strain (designated PYC1\(^{WT}\)). Moreover, PYC113 cells failed to grow on methanol, like *H. polymorpha* pyc1 cells. Western blot analysis revealed that HpPyc113 protein levels were slightly reduced in comparison to the PYC1\(^{WT}\) control (Fig. 5a). Enzyme activity measurements demonstrated that the specific AO activities in cell-free extracts of PYC113 cells (0.06 U mg\(^{-1}\) protein) were very low, as in *pyc1* cells, confirming the AO assembly defect in PYC113 cells.

Next, we analyzed the oligomeric state of AO protein in PYC113. In *pyc1* cells, AO monomers accumulate, whereas in wild-type cells, the majority of the AO protein is octameric (Ozimek *et al.*, 2003). Western blot analysis of fractions...
obtained after sucrose density centrifugation (Fig. 5b) showed that the majority of AO protein in PYC113 cells sedimented to fractions 2, 3 and 4, which correspond to monomeric AO (Goodman et al., 1984), whereas in PYC1WT, AO was present in fractions 7, 8 and 9, the position of AO octamers (Goodman et al., 1984). Immunocytochemical analysis revealed that in PYC113 cells, AO protein was mislocalized to the cytosol, as was observed for pyc1 cells (data not shown; Ozimek et al., 2003).

Taken together, these data demonstrate that the pentapeptide insertion that had occurred in HpPyc113p abolished the function of the protein in AO assembly. Because PYC113 cells are not auxotrophic for aspartate, HpPyc113p is apparently still enzymatically active.

**HpPyc113p and AO physically interact**

Previously, we have shown that HpPyc1p physically interacts with AO protein (Ozimek et al., 2003). We were therefore interested in determining whether the pentapeptide insertion in HpPyc113p influenced the affinity of the protein for AO. To this end, His6-tagged versions of both Pyc1 proteins were produced in *E. coli* and affinity purified. The purified HpPyc1p and HpPyc113p were loaded onto columns containing immobilized AO or BSA, used as a control. The columns were washed with 20 column volumes of wash buffer (F; flow through). Bound proteins were eluted using a buffer containing 8 M urea (E, elution). Equal portions of Input, F and E were subjected to SDS-PAGE and analyzed by Western blotting. Blots were probed with anti-HpPyc1p antibodies. Using wild-type HpPyc1p (left panel), a significant portion of the protein was recovered in fraction E, whereas no HpPyc1p was found in the elution fractions (E) using BSA-containing control columns. Essentially the same result was obtained using mutant HpPyc113p (right panel), with the exception that less protein was recovered in the E and F fractions, probably due to protein instability.

**Fluorescence and CD analysis**

To analyze whether the mutation present in HpPyc113p caused major changes in the protein conformation, we used biophysical approaches, namely tryptophan (Trp) fluorescence analysis and CD.

The fluorescence of Trp residues is sensitive to local environmental changes that may be caused by changes in the tertiary structure of a protein. These changes can result in a shift of the spectral maximum of fluorescence emission, as well as in the Trp fluorescence quantum yield (Lakowicz, 1999). HpPyc1p and HpPyc113p contain eight Trp residues. Fluorescence emission spectra were obtained from purified HpPyc1p and HpPyc113p using excitation at 295 nm (Fig. 7). The spectrum of HpPyc1p shows a fluorescence emission maximum at 327 nm, which indicates that most Trp residues are buried in the hydrophobic interior of the protein. Purified HpPyc113p, however, shows a maximum at 333 nm, in conjunction with a decrease in fluorescence intensity of about 20% relative to the spectrum of wild-type HpPyc1p. The shift of the fluorescence maximum and the drop in quantum yield indicated that the environment of some of the Trp residues in HpPyc113p had become less hydrophobic.

Furthermore, the secondary and tertiary structure of both HpPyc1 proteins were analyzed by CD spectroscopy. Highly similar far-UV spectra were observed for both HpPyc1p and HpPyc113p (data not shown), indicating that the mutation caused no major changes in the secondary structure of the protein. The near-UV spectra (not shown) indicated a slight difference between the two proteins and supported the observed in the control experiment using wild-type HpPyc1p, suggesting that purified HpPyc113p is relatively unstable in vitro or forms aggregates that are not eluted from the column.
fluorescence results, implying that Trp residues are repositioned in the mutant protein to a more polar environment.

Taking these data together, we can conclude that the mutation in HpPyc1<sup>13</sup>p slightly affects the tertiary structure but has no major effect on the secondary structure of the protein. Possibly, the mutant protein has a more open conformation, resulting in more polar environments of some of the Trp residues.

**The TC domain is important for the function of HpPyc1p in AO assembly**

In order to further dissect the region in HpPyc1p that is important for AO assembly, we constructed a series of truncated genes encoding HpPyc1p variants containing different N-terminal and C-terminal deletions. In addition, we analyzed the mutation present in the original HpPYC1 mutant (ass3–110), which showed a defect in AO assembly (Ozimek et al., 2003).

Sequencing of the HpPYC1 gene of mutant ass3–110 revealed a substitution of cytosine at position 1456 with thymine that leads to the introduction of a stop codon [Fig. 8a; HpPyc1<sup>1–485(M)</sup>]. Western blot analysis confirmed the presence of a truncated HpPyc1p form of the expected molecular mass (c. 53 kDa; Fig. 8b). This N-terminal portion of HpPyc1p contains the complete BC domain plus a small part of the region linking the BC and TC domains (Fig. 8a). Because this strain is unable to grow on methanol and showed an AO assembly defect (Table 4), the BC domain of HpPyc1p is apparently not able to activate AO.

In mutant PYC1<sup>13</sup>, a mutation had occurred in the region between the BC and TC domains. To find whether this part of the protein was sufficient for AO assembly, we tested a construct encoding the BC domain plus the linking domain (HpPyc1<sup>1–560p</sup>) or a larger region including also part of the TC domain (HpPyc1<sup>1–815p</sup>). This resulted in the production of the expected truncated proteins in <i>pyc1</i> cells, as
domains is important for AO assembly. The TC domain, the linking region between the BC and TC cells (Table 4). This led us to conclude that in addition to the polymorpha pyc1 enzyme activities similar to that detected in HpPyc1p did not grow on methanol, and showed AO lacking the entire BC domain (HpPyc1480–1175p). Synthesis BC and TC domains alone is not sufficient for AO assembly. control cells (Table 4). Hence, the region between the pyc1 also did not show enhanced AO enzyme activity relative to both strains failed to grow on methanol plus aspartate, and (HpPyc1561–1175p) or with only a portion of the linking region (HpPyc1512–1175p). Both proteins were produced and contained the TC domain without the linking region (HpPyc1480–1175p). Both proteins were produced and showed AO enzyme activities similar to that detected in pyc1 control cells (Table 4). This led us to conclude that in addition to the BC and TC domains is most likely important for AO assembly. Therefore, the defect in AO assembly is not higher (15 and 16) HpPyc1p protein levels than are required for AO assembly. The data from the transposon mutagenesis studies pointed to a dispensable role of the N-terminal BC domain and the C-terminal BCC domain of HpPyc1p. Most insertions that occurred in these regions had no effect on the capacity of the cells to grow on methanol. In line with this, deletion of neither the BC domain nor the BCC domain affected methanol growth.

The BC domain of Pycp proteins contains an ATP-grasp fold (Galperin & Koonin, 1997). Our finding that the BC domain is not necessary for AO assembly suggests that it is unlikely that HpPyc1p is directly involved in donating FAD to AO, because the BC domain is the only domain in HpPyc1p that might be capable of binding FAD. Also, the function of HpPyc1p in AO assembly apparently does not require ATP. This excludes a role of HpPyc1p as a classical chaperone protein like Hsp60 or Hsp70, which are ATPases (Hartl & Hayer-Hartl, 2002).

### Table 4. Growth properties and specific AO activities in different Hansenula polymorpha strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD&lt;sub&gt;660 nm&lt;/sub&gt;</th>
<th>AO activity (U mg&lt;sup&gt;−1&lt;/sup&gt; protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYC1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>3.4</td>
<td>3.83</td>
</tr>
<tr>
<td>pyc1</td>
<td>0.5</td>
<td>0.02</td>
</tr>
<tr>
<td>as2–110 [PYC1&lt;sup&gt;1–485&lt;/sup&gt; (KX)]</td>
<td>0.6</td>
<td>0.09</td>
</tr>
<tr>
<td>PYC1&lt;sup&gt;1–560&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.10</td>
</tr>
<tr>
<td>PYC1&lt;sup&gt;1–815&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.00</td>
</tr>
<tr>
<td>PYC1&lt;sup&gt;480–1061&lt;/sup&gt;</td>
<td>2.8</td>
<td>1.59</td>
</tr>
<tr>
<td>PYC1&lt;sup&gt;480–1175&lt;/sup&gt;</td>
<td>1.9</td>
<td>1.57</td>
</tr>
<tr>
<td>PYC1&lt;sup&gt;512–1175&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.05</td>
</tr>
<tr>
<td>PYC1&lt;sup&gt;561–1175&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Strains of H. polymorpha producing different truncated versions of Pyc1p were grown in mineral media containing methanol as sole carbon and energy source, in the presence of aspartate. The final OD (expressed as OD at 660 nm) was measured after 40 h of incubation. For AO enzyme activity measurements, cell extracts were prepared from cells grown for 24 h on a mixture of methanol and glycerol in the presence of aspartate.

We next analyzed an N-terminal truncated HpPyc1p, lacking the entire BC domain (HpPyc1<sup>480–1175</sup>p). Synthesis of this protein in H. polymorpha pyc1 cells complemented the methanol growth defect paralleled by AO assembly (Table 4). On the basis of these data, we conclude that the BC domain is redundant for the function of HpPyc1p in AO assembly.

We also studied the importance of the BCC domain. The pentapeptide insertions in this region did not affect methanol growth (Fig. 2). As shown in Table 4, pyc1 cells producing HpPyc1<sup>480–1061</sup>p were able to grow on methanol and showed AO enzyme activity; hence, like the BC domain, the BCC domain is redundant for AO assembly.

To further investigate the role of the region between the BC and TC domains, we finally tested two constructs that contained the TC domain without the linking region (HpPyc1<sup>561–1175</sup>p) or with only a portion of the linking region (HpPyc1<sup>512–1175</sup>p). Both proteins were produced and present at the expected molecular mass (Fig. 8b). Hansenula polymorpha pyc1 cells producing these truncated versions of HpPyc1p did not grow on methanol, and showed AO enzyme activities similar to that detected in pyc1 control cells (Table 4). This led us to conclude that in addition to the TC domain, the linking region between the BC and TC domains is important for AO assembly.

### Discussion

In this study, we aimed to delineate the region in H. polymorpha pyruvate carboxylase (HpPyc1p) that is required for the function of HpPyc1p in assembly of peroxisomal AO.

We used a transposon mutagenesis approach that has been successfully used before by others (Hallet et al., 1997; Fransen et al., 2005). There are multiple advantages of this approach, such as: (1) random distribution of mutations; (2) the possibility of selecting mutant genes containing one insertion per gene; (3) easy selection of mutated genes; (4) more severe effects than are obtained by classical PCR-based mutagenesis, because of the insertion of a pentapeptide; and (5) no introduction of frameshifts or stop codons.

The mutagenized HpPYC1 genes were transformed to H. polymorpha pyc1 cells that are Asp<sup>−</sup> Mut<sup>−</sup>. Approximately half of the transformants showed a wild-type phenotype (Asp<sup>+</sup> Mut<sup>+</sup>). Four transformants were Asp<sup>−</sup> Mut<sup>−</sup>. In three of them (numbers 14, 15 and 16), insertions had occurred in the central TC domain. These strains contain similar (14) or higher (15 and 16) HpPyc1p protein levels than are required for AO assembly. Therefore, the defect in AO assembly is not due to too low HpPyc1p protein levels. Hence, the TC domain is most likely important for AO assembly, in addition to its function in the transfer of a carboxyl group from biotin to pyruvate (Attwood & Wallace, 2002). Two strains (PYC1<sup>17</sup> and PYC1<sup>18</sup>) carrying pentapeptide insertions in the C-terminal part of the TC domain had only a minor effect on the ability of the cells to grow in the absence of aspartate, whereas no defect in methanol growth was detected. These mutations, therefore, most likely did not significantly disturb the function of the TC domain of HpPyc1p.
occurred in this region (Pyc113) did not result in aspartate auxotrophy, but fully abolished its function in AO assembly, suggesting an important role for this region in AO assembly. In line with this conclusion is the finding that the N-terminal truncated proteins that lacked this domain completely (561–1175) or partially (512–1175) were not able to mediate AO assembly.

Alignment of the region that links the BC and TC domains (amino acids 480–560 in HpPyc1p; Fig. 9) with Pycp proteins from other eukaryotes revealed a highly conserved part (amino acids 480–511) followed by a less conserved region (amino acids 512–560). The pentapeptide insertion that occurred in HpPyc113p is present in the conserved region (amino acids 512–560). The pentapeptide core motif composed of eight parallel \( \alpha \)-helices (Banner et al., 1975). The C-terminal truncated HpPyc113–1175p contains the complete \( \beta_6\alpha_6 \) core of the TC domain. However, only very minor amounts of this truncated version of HpPyc1p were observed (Fig. 8b), despite the fact that it was expressed under control of the strong \( P_{AOX} \). Probably, all structural elements in the HpPyc1p TC domain are required to form a stable conformation. This is supported by the observation that several pentapeptide insertions in the TC domain resulted in relatively low protein levels (Fig. 3).

Because deletions in the region between the BC and TC domains also resulted in low levels of HpPyc1p (in HpPyc1512–1175p and HpPyc1561–1175p), this region of the protein could be part of the highly structured TC domain in eukaryotic Pyc proteins as well. This is also suggested by data obtained by Lim et al. (1988), who showed that upon limited proteolysis of \( S. \) cerevisiae Pyc, a fragment was obtained that represented the TC domain, including the linking region (residues 473–967 in ScPyc, corresponding to HpPyc residues 477–971).

How the central domain of HpPyc1p functions in assisting FAD binding to AO apoprotein is still unknown. Possibly, HpPyc1p binds to newly synthesized AO monomers and stabilizes a conformation that allows FAD binding. Detailed in vitro reconstitution experiments may shed further light on this intriguing phenomenon.

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