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Published in:
Fems Yeast Research

DOI:
10.1111/j.1567-1364.2010.00647.x

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Activation of a peroxisomal *Pichia pastoris* d-amino acid oxidase, which uses d-alanine as a preferred substrate, depends on pyruvate carboxylase

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Received 7 January 2010; revised 5 May 2010; accepted 7 May 2010. Final version published online 9 June 2010.

DOI: 10.1111/j.1567-1364.2010.00647.x

Editor: André Goffeau

**Keywords**
d-amino acid oxidase; peroxisome; pyruvate carboxylase; *Pichia pastoris*; moonlighting.

**Abstract**
D-Amino acid oxidase (DAO) is an important flavo-enzyme that catalyzes the oxidative deamination of d-amino acids into the corresponding α-keto acid, ammonia and H2O2. We identified two amino acid oxidases in the methylotrophic yeast *Pichia pastoris*: Dao1p, which preferentially uses d-alanine as a substrate, and Dao2p, which uses d-aspartate as a preferred substrate. Dao1p has a molecular mass of 38.2 kDa and a pH optimum of 9.6. This enzyme was localized to peroxisomes, albeit a typical peroxisomal targeting signal is lacking. Interestingly, *P. pastoris* mutant strains, defective in the enzyme pyruvate carboxylase, showed a pronounced growth defect on d-alanine, concomitant with a significant reduction in Dao1p activity relative to the wild-type control. This indicates that pyruvate carboxylase functions in import and/or activation of *P. pastoris* Dao1p.

**Introduction**
D-Amino acid oxidase (DAO, EC 1.4.3.3) was first described in 1935, and since then, has become a model enzyme for the dehydrogenase-oxidase family of flavoproteins (Krebs, 1935). It catalyzes the oxidative deamination of d-amino acids to the corresponding α-keto acid with a strict stereospecificity. A noncovalently bound FAD moiety is used as an electron acceptor, which reoxidizes via molecular oxygen to produce H2O2. DAO is predominantly observed in eukaryotes; however, one bacterial DAO was characterized recently (Geueke et al., 2007).

Industrially relevant applications for DAO are the production of α-keto acids and pure L-amino acids, the analytical determination of d-amino acids and especially the production of the cephalosporin antibiotics precursor 7-aminocephalosporanic acid. More than 50 different semisynthetic cephalosporins are synthesized from this antibiotic agent by modification of the side chain (reviewed in Pollegioni et al., 2008).

DAO contains a peroxisomal targeting signal 1 (PTS1) in various eukaryote organisms and is imported into peroxisomes by the soluble receptor Pex5p (Yurimoto et al., 2000). Peroxisomes are ubiquitous and single-membrane-bound organelles. The physiological function, protein composition and abundance of peroxisomes strongly depend on the organism or the cell type in which they occur and may alter with changes in environmental conditions (van der Klei & Veenhuis, 2006). The majority of peroxisomal matrix proteins are imported via the PTS1. The signal consists of a tri-peptide at the extreme C-terminus with the consensus sequence (S/A/C)-(K/R/H)-L, and is recognized by the soluble import receptor Pex5p (Swinkels et al., 1992; McCollum et al., 1993). However, Pex5p is also required for import of the so-called ‘non-PTS’ proteins that either lack a recognizable targeting signal or contain a signal that is redundant for import (Klein et al., 2002; Gunkel et al., 2004).

In methylotrophic yeast, the activation of another member of the dehydrogenase-oxidase family, alcohol oxidase...
(AO), depends on the chaperone-like activity of the cytosolic protein pyruvate carboxylase (Pyc1p) (Ozimek et al., 2003). Pyc1p replenishes the citric acid cycle with oxaloacetate, but contains an additional (moonlighting) function in the activation pathway of AO that is independent of its enzyme activity. Like DAO, AO also is a peroxisomal and flavin-containing enzyme. In pyc1-deficient strains of Hansenula polymorpha and Pichia pastoris, AO is mislocalized to the cytosol as a monomer that lacks FAD.

Here, we report the presence of two DAO homologs in the yeast P. pastoris: Dao1p, which preferentially uses D-alanine as a substrate, and Dao3p, which has D-aspartate as a preferred substrate.

Detailed analysis of Dao1p revealed that it has a pH optimum of 9.6. This enzyme was localized to peroxisomes despite the absence of a typical peroxisomal targeting signal. Interestingly, cells of a pyruvate carboxylase-deficient strain showed a growth defect on media containing D-alanine and L-aspartate (60 mg L

Materials and methods

Organisms and culture conditions

The P. pastoris strains used in this study are listed in Table 1. All strains were cultured at 30 °C. The media components were as follows: solid media contained 0.17% yeast nitrogen base, 2.2% agar, pH 6.0, with either 0.5% glucose or 0.3% ethanol in the presence of 0.5% ammonium sulfate. Oleate plates contained 0.1% oleic acid, 0.5% Tween 80, 0.1% yeast extract, 0.5% ammonium sulfate, 0.17% yeast nitrogen base and 2.2% agar, pH 6.0. D-Alanine plates contained 0.6% D-alanine, 0.5% glucose, 0.17% yeast nitrogen base and 2.2% agar, pH 6.0. Liquid mineral medium (van Dijken et al., 1976) was supplemented with 0.5% glucose and 0.25% ammonium sulfate or 0.5% methanol with either 0.25% ammonium sulfate or 0.25% D-alanine. Histidine, arginine and L-aspartate (60 mg L

Table 1. Pichia pastoris strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP36 his3</td>
<td>Menendez (1998)</td>
</tr>
<tr>
<td>MP36 his3, pyc1::HIS3</td>
<td>Menendez (1998)</td>
</tr>
<tr>
<td>PYP12 his4, arg4, RPPA05600::zeocin</td>
<td>This study</td>
</tr>
<tr>
<td>PYP12 his4, arg4, RPPA06337::hygromycin</td>
<td>This study</td>
</tr>
<tr>
<td>PYP12 his4, arg4, HIS4::pPIC3.5K-DAO</td>
<td>This study</td>
</tr>
</tbody>
</table>

Identification of PpDAO

A BLAST search of the P. pastoris genome database (Integrated Genomics, Chicago) revealed two homologs, RPPA05600 and RPPA06337, of human D-amino acid oxidase (HsDAO). Based on homology to known genes and its function (see Results), RPPA05600 was designated as PpDAO1.

Construction of deletion strains

The 0.2 kbp 5′ flanking region of RPPA05600 was amplified from PPy12 genomic DNA using the primers SAK50 and SAK51 (Table 2). The 0.2 kbp 3′ flanking region of RPPA05600 was amplified using oligos SAK52 and SAK53. Both fragments were cloned into pBS-ARPPA05600. The plasmid pBS-zeo was constructed in the following way: the 1.1 kbp zeocin cassette was amplified from pREMI-Z (van Dijk et al., 2001) using the primers Zeo-XbaI-FW and Zeo-XhoI-RV and cloned into the XbaI/Xhol fragment of pBluescript II KS+(Stratagene, La Jolla, CA). The deletion cassette was amplified from pBS-ARPPA05600 with SAK50 and SAK53 and used to transform PPy12. Transformants were selected for their ability to grow in the presence of zeocin and checked by Southern blotting.

The 0.4 kbp 5′ flanking region of RPPA06337 was amplified from PPy12 genomic DNA with the primers SAK54 and SAK57. Both fragments were cloned into pBS-del-PEX3-hph to obtain pBS-del-PEX3-hph was constructed in the following way: the SacI site of pAG32 (Goldstein & McCusker, 1999) was removed by digestion, T4 DNA polymerase blunt ending and relegation, yielding pAG32B. In the second step, a 1.7 kbp BglII/EcoRV fragment of pAG32B was ligated to a 3.6 kbp BglII/EcoRV vector fragment of pRBG3 (Baerends et al., 2000). The deletion cassette was amplified from pBS-ARPPA06337 with SAK54 and SAK57 and used to transform PPy12. Transformants were selected for their

Table 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAK50</td>
<td>GGGGAGCTCATCCTCAAAATCCCACCTG</td>
</tr>
<tr>
<td>SAK51</td>
<td>CCAAGCGGGCGCCGGCTTTGGCAGTTCAGAG</td>
</tr>
<tr>
<td>SAK52</td>
<td>GGCTCGAGCTCCAAACAGATAGCTCATAC</td>
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<tr>
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<td>SAK54</td>
<td>GGATGTCGACCTTGGGCTGTCATCTTC</td>
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<tr>
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<td>GCCGAGATCTTGTGGGATGACCTTCTTC</td>
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<tr>
<td>SAK56</td>
<td>GAGATGTCGCCATCCCTTGCATCTGCG</td>
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<tr>
<td>SAK57</td>
<td>CAGAGCTCAGCTCTCGTGAATTTTTGG</td>
</tr>
<tr>
<td>SAK59.1</td>
<td>CCAGGATCCATGACGGTTGCAACCCATTTATCATTGG</td>
</tr>
<tr>
<td>SAK95.1</td>
<td>CCAGGATCCATGACGGTTGCAACCCATTTATCATTGG</td>
</tr>
<tr>
<td>Zeo-XbaI-FW</td>
<td>CGCTCGAGCTCCTGCTGAAATTTTTGG</td>
</tr>
<tr>
<td>Zeo-XhoI-RV</td>
<td>CGCGGATCCCATGACGGTTGCAACCCATTTATCATTGG</td>
</tr>
</tbody>
</table>

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ability to grow in the presence of hygromycin and checked by Southern blotting.

**Ectopic expression**

The full-length DAO1 ORF was amplified from PYY12 genomic DNA using the primers SAK94.1 and SAK95.1 and expressed from the AOX promoter in the vector pPIC3.5K (Invitrogen, Breda, the Netherlands). The resulting plasmid, designated pPIC3.5K-DAO, was linearized with Sall and transformed to PYY12 cells. Transformants were selected by histidine prototrophy and confirmed by Southern blotting.

**Molecular techniques**

The oligonucleotides used in this study are presented in Table 2. All standard DNA procedures were performed as described (Sambrook et al., 1989). Electro-transformation was performed according to Faber et al. (1994).

**Biochemical methods**

DAO- and d-aspartate oxidase-specific enzyme activities were measured spectrophotometrically at 30 °C in crude cell extracts essentially as described previously (Sulter et al., 1990). FAD (5 μM) was added during the reaction, and 50 mM d-alanine and 10 mM d-aspartate, respectively, were used as a substrate. Protein concentrations were determined using the Bio-Rad assay kit and bovine serum albumin as a standard (Bio-Rad GmbH, Munich, Germany). For determination of the pH optimum, 100 mM sodium carbonate–bicarbonate buffer was utilized that buffers well between pH 8.5 and 11.5.

**Electron microscopy**

Protoplasts were generated essentially as described by Faber et al. (1998), with the exception that cells were treated with 10 mM DTT before protoplasting was performed in the presence of 2.4 M sorbitol. Fixation, cytchemical staining and electron microscopy of protoplasts were performed according to Veenhuis et al. (1976).

**Results**

**Identification of two DAOs in P. pastoris**

In order to search for DAO homologs of *P. pastoris*, the gene coding for HsDAO was used as a query against the *P. pastoris* genome database. Two genes, RPPA05600 and RPPA06337, which code for two proteins of 344 and 404 amino acids, respectively, were identified. RPPA05600 was designated DAO1 and RPPA06337 was named DAO2. A database search revealed that the protein product of DAO1, Dao1p, shows the highest homology to *Candida boidinii* DAO (66% identity). Dao2p is 38% identical to CbDAO (Fig. 1). Both proteins contain the N-terminal GxGxxG nucleotide-binding sequence that relates to the presence of a Rossman fold in the protein structure, a common structural feature of flavin-containing oxidoreductases. Neither Dao1p nor Dao2p contains a peroxisomal targeting signal type 1 or 2.

**Dao1p exhibits d-alanine oxidase activity**

DAO1 and DAO2 deletion strains were constructed in the *P. pastoris* PYY12 background and analyzed for defects in the utilization of d-alanine (Fig. 2) using a spot assay. Serial dilutions of cells were spotted on YND plates containing glucose in the presence of d-alanine as the sole nitrogen source. The data presented in Fig. 2a show that cells of the Adao1 strain display a strong growth defect, relative to Adao2 and the wild-type (WT) (PYY12) control. This growth defect was accompanied by a significant reduction in the d-alanine oxidase-specific enzyme activity, detected in crude extracts prepared from d-alanine-grown Dao1p-deficient cells (Fig. 2b). Identically grown cells of the Adao2 strain contained normal DAO activities for d-alanine comparable to those in WT controls. To confirm that the d-alanine oxidase activity is exhibited by the product of the DAO1 gene, DAO1 was overexpressed under control of the strong AO promoter. Cells of the transformants were grown on mineral media supplemented with methanol as the sole carbon source to induce the synthesis of Dao1p. The d-alanine oxidase-specific activities detected in crude cell extracts of these cells amounted to ~2.3 U mg⁻¹ protein, which is about 120 times higher than the endogenous WT activities.

The pH optimum of the enzyme was determined using crude extracts of Dao1p-overproducing cells (Fig. 3). The enzyme showed a pH optimum of pH = 9.6. The pH optimum appeared to be rather broad and the enzyme functions well between pH 9.1 and 10.6.

Subsequently, peptide antibodies were generated against Dao1p. These antibodies recognized a specific protein band in Western blots, prepared from crude extracts of the DAO1 overexpression strain that was absent in the Adao1 strain (data not shown). However, in PYY12 WT cells, no protein band was detected. From this, we conclude that Dao1p is present at low levels in WT cells, consistent with the relatively low specific enzyme activities.

**Dao2p exhibits d-aspartate oxidase activity**

Dao1p and Dao2p share approximately 35% sequence identity with human d-aspartate oxidase. To determine whether one of the proteins displays d-aspartate oxidase activity, the WT strain PYY12 and the Adao1 and Adao2 strains were grown on glucose in the presence of d-aspartate as the sole nitrogen source (Fig. 4). Enzyme measurements...
using crude extracts of these cells demonstrated that the WT host and \( D_{\text{dao1}} \) cells contained comparable amounts of \( D \)-aspartate oxidase activity, whereas the enzyme activity was below the limit of detection in extracts of \( D_{\text{dao2}} \) cells.

**Dao1p is localized to peroxisomes**

All DAOs identified so far contain a C-terminal PTS1 and have been localized to peroxisomes (Perotti \textit{et al}., 1987; Perotti \textit{et al}., 1991). The C-terminal residues of Dao1p, -AAK, do not match the PTS1 consensus. Accordingly, neither a PTS1 (Neuberger \textit{et al}., 2003) nor the PTS2 predictor (Schlueter \textit{et al}., 2007) recognized the presence of a known PTS in Dao1p. As the low Dao1p levels did not allow detection by immunocytochemistry using the anti-Dao1p antibodies, we aimed to determine the subcellular location of the enzyme using the very sensitive cerium-based cytochemical method (Veenhuis \textit{et al}., 1976). This method is based on the trapping of the \( H_2O_2 \) side product generated by the enzyme at the site of its location. Incubation of protoplasts prepared from cells of the WT (PPY12) strain in the presence of \( CeCl_3 \) and \( D \)-alanine resulted in a cerium reaction product that was confined to the peroxisomal matrix (Fig. 5). This convincingly demonstrates that in WT cells, Dao1p activity is confined to the peroxisomal matrix.

**Growth on \( D \)-alanine and Dao1p enzyme activity are decreased in a \( D_{\text{pyc1}} \) strain**

Previous reports showed that pyruvate carboxylase (Pyc1p) is required for the import and assembly of the peroxisomal flavo-enzyme AO in the yeasts \( H. \) polymorpha and \( P. \) pastoris (Ozimek \textit{et al}., 2003). This led us to investigate whether Pyc1p was also required for Dao1p activation. First, we analyzed the growth properties of a \( P. \) pastoris \( PYC1 \) deletion strain (\( D_{\text{pyc1}} \)) on various carbon and nitrogen sources. The data depicted in Fig. 6a show that a strong reduction in growth was observed for \( D_{\text{pyc1}} \) on glucose/\( D \)-alanine. However, \( D_{\text{pyc1}} \) cells of \( P. \) pastoris have to be supplemented with aspartate or glutamate to facilitate the growth.
growth on glucose in liquid media (Stucka et al., 1991; Brewster et al., 1994). On ethanol, however, cells grow normally due to the induction of a bypass pathway, the glyoxylate cycle, which complements for the withdrawal of intermediates from the TCA cycle. Therefore, additional growth experiments were performed in ethanol-containing liquid media supplemented with various nitrogen sources to assess whether the growth defect of a Δpyc1 strain on D-alanine was specific for this nitrogen source (Fig. 6b). Δpyc1 cells grew like WT on ethanol/(NH₄)₂SO₄ (data not shown) and ethanol/L-aspartate. However, the growth of Δpyc1 cells on ethanol/D-alanine is retarded, consistent with the growth data obtained on solid plates.

The observed defect of Δpyc1 to grow on ethanol/D-alanine was associated with a strong decrease (approximately 70%) in Dao1p-specific enzyme activity relative to the activity detected in the WT host strain (Fig. 6c).

**Fig. 2.** Dao1p exhibits DAO activity. (a) Spot assay of Pichia pastoris WT (PPY12), Δdao1 and Δdao2 on solid media containing glucose and 0.6% D-alanine. Cells were grown for 3 days at 30°C. (b) Dao1p-specific enzyme activities, determined in crude extracts of glucose/D-alanine cultured cells. The activities in extracts of Δdao1-deficient cells are strongly decreased. Overexpression of DAO1 under control of the inducible AOX promoter increases Dao1p enzyme activities to 2.3 U mg⁻¹ protein. The mean value of two independent experiments is shown.

**Fig. 3.** Dao1p has an alkaline pH optimum. The specific activity of Dao1p was determined using extracts of WT PPY12 cells that overexpress DAO1. Dao1p displays the highest activity between pH 9.5 and 10.0.
strongly suggests that in the absence of Pyc1p, normal assembly/activation of Dao1p is significantly disturbed.

Discussion

This paper describes the identification of two DAOs from the yeast \textit{P. pastoris}: Dao1p and Dao2p. The first enzyme prefers \(\alpha\)-alanine as a substrate, is a peroxisomal enzyme, and like peroxisomal AO (Ozimek et al., 2003), is dependent on pyruvate carboxylase for activation. Microbial DAOs from \textit{Fusarium oxysporum}, \textit{Candida para-}

psilosis (Gabler et al., 2000) and \textit{C. boidinii} (Yurimoto et al., 2001) have a high specificity toward \(\alpha\)-alanine, whereas DAO from \textit{Rhodotorula gracilis} (Gabler et al., 2000), \textit{Trigonopsis variabilis} (Schrader & Andreesen, 1996) and \textit{Verticillium luteoalbum} (Gabler et al., 2000) display a preference toward \(\alpha\)-methionine. \textit{Pichia pastoris} Dao1p was active toward \(\alpha\)-alanine, but not toward \(\alpha\)-methylamine (data not shown). This is consistent with data for \textit{C. boidinii} DAO that is highly homologous with \textit{P. pastoris} DAO. The activity of \textit{C. boidinii} DAO toward \(\alpha\)-methionine is also 86\% lower relative to \(\alpha\)-alanine (Yurimoto et al., 2001). The pH optimum of \textit{P. pastoris} Dao1p (pH = 9.6) is within the range that has been described for DAOs from other sources (Yurimoto et al., 2001). Earlier work suggested that \(\alpha\)-amino acids with a deprotonated amino group are better substrates for DAOs (Harris et al., 2001). The pH value for the \(\alpha\)-amino group proton dissociation amounts to 9–9.5 and matches the pH optimum of \textit{P. pastoris} Dao1p. Lower pH optima (e.g. \textit{T. variabilis}) have been explained by the change in the microenvironment upon binding of the substrate (Harris et al., 2001).
Unlike other DAOs, the *P. pastoris* Dao1p enzyme does not contain a peroxisomal targeting signal, but it is nevertheless a bona fide component of the peroxisome matrix. This suggests that Dao1p belongs to the family of non-PTS proteins that require the PTS1 receptor Pex5p for import (Klein et al., 2002; Gunkel et al., 2004). The actual targeting of such proteins is still unclear, but evidence is emerging that it is in fact a conformational signal rather than a linear epitope that is recognized by the receptor. Whether Dao1p is imported via this alternative route still has to be demonstrated.

Alternatively, the PTS1 recognizing TPR domain of *P. pastoris* Pex5p could have evolved to recognize a different and species-dependent PTS1 consensus and bind to – AAK, the extreme C-terminus of Dao1p. Koller et al. (1999) showed that the C-terminus of acyl CoA oxidase (Pox1p), PKI, which does not fit the PTS1 consensus, directs a reporter protein to peroxisomes. However, Elgersma et al. (1996) showed that there is a strong requirement for a hydrophobic residue at the ultimate position of the PTS1. The isoleucine of the Pox1p PTS1 fulfills this requirement, but not the C-terminal lysine of Dao1p. Therefore, it remains questionable whether – AAK could represent a targeting signal for Dao1p.

A possible alternative for the import of Dao1p is that it forms heterooligomers with a PTS1-containing protein. An example of this has been shown recently in higher eukaryotes, where the enzyme Cu/Zn superoxide dismutase is imported into peroxisomes, together with its PTS1 carrying interaction partner copper chaperone (Islinger et al., 2009).

The physiological role of DAO in eukaryotes is diverse. It plays a catabolic role in lower eukaryotes that allow the organisms to use d-amino acids as carbon and nitrogen sources (Yurimoto et al., 2000). In the human brain, DAO plays a regulatory role and controls the levels of the neurotransmitter serotonin. Of interest is the implication of DAO in the pathophysiology of schizophrenia (reviewed in Verrall et al., 2009). A genetic association was observed between the mammal-specific gene G72 and schizophrenia. Yeast two-hybrid studies revealed an interaction between G72 and DAO, and *in vitro* experiments showed an increased enzyme activity of DAO in the presence of G72. However, some unsolved issues remain, such as the different intracellular localizations of G72 and DAO (Chumakov et al., 2002).

Surprisingly, *P. pastoris* cells deficient of the anaerocytic enzyme Pyc1p showed a growth defect in media containing d-alanine as the sole nitrogen source. This defect was specific for d-alanine and due to a reduced enzyme activity of Dao1p. These data lead to the hypothesis that Pyc1p is involved in the activation of *P. pastoris* Dao1p. Pyc1p is also involved in the assembly/activation of peroxisomal AO of methanolotrophic yeast species (Ozimek et al., 2003). Binding of FAD is crucial for import of AO (Evers et al., 1996), and in *Apyc1* cells, AO is mislocalized to the cytosol in its monomeric form and lacks FAD (Ozimek et al., 2003). This raised the hypothesis that Pyc1p aids binding of the FAD cofactor to AO monomers, the step required for oligomerization of AO. PpDao1p is similar to AO in that both are peroxisomal flavo-dependent oxidoreductases and bind FAD via the Rossmann fold. The PTS1 of AO is redundant for import and – like Dao1p – AO belongs to the class of non-PTS proteins. Apparently, Pyc1p has acquired a second function in methanolotrophic yeast to aid the assembly of peroxisomal flavo-proteins.

This phenomenon, termed ‘moonlighting,’ is not restricted to the assembly of peroxisomal proteins, but is observed in many cell functions (Gancedo & Flores, 2008). Multifunctional proteins that have not originated from gene fusion or differential splicing fall into this category. Examples from *S. cerevisiae* are enolase, which catalyzes the interconversion of 2-phosphoglycerate and phosphoenolpyruvate and has a second function in vacuolar protein trafficking (Decker & Wickner, 2006), or Arg83, which is a transcriptional regulator and inositol phosphokinas (Saiardi et al., 1999; Odom et al., 2000). Studies to unravel the molecular principles of the function of Pyc1p in the activation of Dao1p and AO are ongoing.

**References**


