The significance of peroxisomes in methanol metabolism in methylotrophic yeast

Ida J. van der Klei\textsuperscript{a}, Hiroya Yurimoto\textsuperscript{b}, Yasuyoshi Sakai\textsuperscript{b}, Marten Veenhuis\textsuperscript{a,\dagger}

\textsuperscript{a} Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, NL-9750 AA Haren, The Netherlands

\textsuperscript{b} Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan

Received 30 April 2006; accepted 26 July 2006
Available online 1 September 2006

Abstract

The capacity to use methanol as sole source of carbon and energy is restricted to relatively few yeast species. This may be related to the low efficiency of methanol metabolism in yeast, relative to that of prokaryotes. This contribution describes the details of methanol metabolism in yeast and focuses on the significance of compartmentalization of this metabolic pathway in peroxisomes.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Peroxisome; Yeast; Methanol metabolism

1. Methylotrophy in prokaryote and eukaryote micro-organisms

A limited number of yeast species is capable to grow on methanol as sole source of carbon and energy. To enable this, these organisms use highly specialized metabolic pathways that are partly compartmentalized in peroxisomes. Consequently, peroxisomes are abundantly present in methanol-grown cells but strongly reduced in number in cells in which methanol utilization is repressed (see Fig. 1). Organisms that are capable to grow on C1 compounds, like methanol, are designated methylotrophs. These organisms share the remarkable capacity to derive all energy and cell carbon from reduced molecules that have no C–C bond. Methylotrophy is confined to a few prokaryote and eukaryote micro-organisms. Prokaryote methylotrophs are capable to grow on a variety of C1 compounds (e.g. methanol, methylamine, methane) whereas in eukaryotes methylotrophy is limited to methanol utilization.

The general mode of methylotrophs to assimilate carbon is to convert three C1 molecules into a C3 compound via a cyclic pathway (like in photosynthesis). In prokaryotes, three different pathways are known (i.e. the ribulose bisphosphate cycle, the ribulose monophosphate cycle and the serine pathway), but in methylotrophic yeast the xylulose monophosphate cycle uniquely operates in C1 assimilation. For the initial oxidation of methanol, prokaryotes and eukaryotes use different enzymes, namely a dehydrogenase or oxidase, respectively. For further details on methylotrophy the reader is referred to a comprehensive overview in \textit{Biochemistry of Methylotrophs} (Anthony, 1982\cite{1}).

Oxidation of the relatively toxic compound methanol in yeast results in the formation of two other very reactive compounds, formaldehyde and hydrogen peroxide. The enzyme that catalyses this reaction, alcohol oxidase (AO) is compartmentalized in peroxisomes, together with catalase (CAT) which decomposes hydrogen peroxide into water and oxygen (Fig. 2A). The presence of hydrogen peroxide producing oxidases and catalase is a characteristic feature of peroxisomes in all eukaryotes and has the advantage that the enzymes that produce and decompose hydrogen peroxide are closely associated, preventing diffusion of this hazardous compound into the cytoplasm \cite{2,3}.

In methylotrophic yeast a third enzyme of methanol metabolism is localized to peroxisomes, namely dihydroxyacetone synthase (DHAS). DHAS is a component of the xylulose-5-phosphate cycle and catalyses the first step of formaldehyde...
assimilation to form two C3 molecules (dihydroxyacetone and glyceraldehyde-3-phosphate) from one C1 (formaldehyde) and one C5 (xylulose-5-P) molecule (Fig. 2A). The peroxisomal localization of AO, CAT and DHAS is essential to allow methylotrophic yeast cells to grow on methanol [4,5]. This characteristic has been an important tool in the isolation of peroxisome-deficient mutants (pex) of methylotrophic yeast species, as such mutants have lost the capacity to utilize methanol despite the fact that they do contain all enzymes involved in methanol metabolism [6,7]. Detailed physiological studies of yeast pex mutants have revealed why compartmentalization is essential for methylotrophic growth of yeast [8] (see Section 4). All other enzymes required for methanol utilization are cytosolic in yeast. These include the enzymes for the oxidation of formaldehyde to CO₂ (formaldehyde dehydrogenase, S-formylglutathione hydrolase, and formate
dehydrogenase) and all enzymes of the xylulose-5-P pathway, except for DHAS [5,9] (Fig. 2).

The first electron micrographs of methanol-grown yeast cells were published in the seventies of the last century (Fig. 1A, C; [10,11]). These amazing images, showing cells crowded with relatively large cubic peroxisomes that occupy almost the entire cytoplasmic volume, have strongly stimulated the interest in peroxisomes and triggered the onset of peroxisome research in yeast.

In this contribution we discuss the principles of methanol metabolism in yeast, focusing on the enzymes, the metabolic pathways and the crucial role of peroxisomes.

![Fig. 2. Schematic representation of methanol metabolism in H. polymorpha WT (A) and pex mutants (B). (A) Schematic representation of the compartmentalization and the function of peroxisomes in methanol metabolism in H. polymorpha WT cells. 1 — alcohol oxidase, 2 — catalase, 3 — formaldehyde dehydrogenase, 4 — S-formyl glutathione hydrolase, 5 — formate dehydrogenase, 6 — dihydroxyacetone synthase, 7 — dihydroxyacetone kinase, 8 — formaldehyde reductase, 9 — glutathione reductase, 10 — oxidation of glutathione. (B) Hypothetical scheme of methanol metabolism in a H. polymorpha pex mutant during growth of cells in a glucose-limited continuous culture, supplemented with methanol as second substrate in the feed. 1 — alcohol oxidase (catalyses both the oxidation of methanol and methylene glycol, the hydrated form of formaldehyde), 2 — formaldehyde dehydrogenase, 3 — S-formyl glutathione hydrolase, 4 — formate dehydrogenase, 5 — glutathione reductase, 6 — NADH/NADPH transhydrogenase, 7 — cytochrome c peroxidase, 8 — catalase, 9 — oxidation of glutathione. Enzymes which are thought to be of minor significance in methanol metabolism in pex mutants (dihydroxyacetone synthase, formaldehyde reductase) are not included [adapted from [8]].]
2. Methylo trophic yeast species

All known methylo trophic yeast species belong to the genera *Pichia*, *Candida* and *Torulopsis*. Best studied species are *P. pastoris*, *P. methanolic a*, *C. boidinii* and *Pichia augusta*, generally referred to as *Hansenula polymorpha*.

Most methylo trophic yeast strains have first been isolated from samples containing decaying plant material. For instance, *H. polymorpha* species have been isolated from an exudate of oak, whereas *H. polymorpha* species have been obtained from spoiled concentrated orange juice. Most likely, decaying materials of plant origin are the natural site for methylo trophic yeast, because methanol is released during hydrolysis of pectin, a major plant constituent [12].

The massive proliferation of peroxisomes in methylo trophic yeast has rendered these micro-organisms very attractive for fundamental research on the molecular mechanisms of peroxisome biogenesis, degradation and function [5–7,13,14]. However, they are also very important for various industrial applications. The most important one nowadays is their use as hosts for the production of heterologous proteins. Methylo trophic organisms are very attractive, because they harbor very strong, inducible promoters (e.g. for expression of the genes encoding AO and DHAS) (for a recent review see [15]). In fact, strong, inducible promoters (e.g. for expression of the genes encoding AO and DHAS) (for a recent review see [15]). In fact, the alcohol oxidase promoter (*P*AOX) is one of the strongest promoters in nature and also one of the most tightly controlled ones (for a recent review see [16]).

Another advantage of the use of methylo trophic yeast as cell factory is that secreted proteins are not hyperglycosylated, a major drawback that is often encountered using *Saccharomyces cerevisiae*. An additional advantage of *H. polymorpha* is that this organism is thermotolerant (it can grow at temperatures up to 47 °C), which is beneficial in large scale fermentations because it reduces cooling costs and microbial contamination [15].

3. Enzymes involved in C1 metabolism in methylo trophic yeast

3.1. The initial steps: Alcohol oxidase and CAT

In yeast, methanol is oxidized by the peroxisomal flavo enzyme AO (EC 1.1.3.13), an enzyme that belongs to the family of glucose–methanol–choline oxidoreductases [16]. The enzyme is designated AO because of its relatively broad substrate specificity as it oxidizes various short aliphatic alcohols in addition to methanol. In vivo, however, AO is only involved in methanol oxidation. For growth of yeast cells on ethanol, not AO but the cytosolic enzyme alcohol dehydrogenase is responsible.

In its enzymatic active form AO consists of eight identical subunits, each of which contains one FAD molecule non-covalently bound as the cofactor. The regions involved in FAD-binding are the best-conserved ones in the primary sequence of AO. The most important one is the common ADP-binding motif (βαβ), which consists of the nucleotide-binding site GXGXXG. So far, no three-dimensional model is available of octameric AO proteins. However, based on the structure of *Aspergillus nidulans* glucose oxidase (GOX), a 3D model has been prepared of *H. polymorpha* AO monomers [17].

The oxidation of methanol by AO requires oxygen and results in the formation of hydrogen peroxide and formaldehyde. Inside the peroxisomes, hydrogen peroxide is dismutated by CAT to oxygen and water. Active peroxisomal CAT protein is a homotetramer of approximately 240 kDa that contains heme as a cofactor.

In methanol-grown cells, AO and DHAS are the most abundant proteins, which – dependent on the growth conditions – can readily reach levels up to 70% of the total cellular protein. At these high expression levels AO protein forms crystalloids, which are responsible for the characteristic cubic shape of peroxisomes in methylo trophic yeast. Within these crystalloids DHAS protein is dispersed (Fig. 1F). The CAT enzyme protein is predominantly located at the periphery of the organelle (between AO crystalloid and peroxisomal membrane; Fig. 1E [18]). This CAT barrier is of major importance to prevent leakage of hydrogen peroxide produced by AO into the cytosol. The importance of CAT is stressed by the observation that CAT deficient *H. polymorpha* cells are unable to grow on methanol [19]. Also in *C. boidinii* methanol growth is strongly affected upon deletion of the CAT gene [20].

3.2. PMP20 glutathione-peroxidase as a peroxisomal antioxidant enzyme

The peroxisomal matrix and membranes are assumed to be exposed to a high level of reactive oxygen species (ROS) because hydrogen peroxide is generated by the oxidase reaction within the organelle. Therefore, as in other ROS generating organelles, such as mitochondria, peroxisomes are assumed to have defensive enzyme activities against ROS toxicity [21]. Obviously, one of these is peroxisomal CAT. In mammalian peroxisomes, two other antioxidant enzymes are also present, i.e. superoxide dismutase [22,23] and glutathione peroxidase (GPX) [24].

In *C. boidinii*, Pmp20 was initially identified as a peroxisomal peripheral membrane protein of an unknown function [25]. *C. boidinii* Pmp20 (ChPmp20), which is associated with the inner side of peroxisomal membrane, belongs to a protein family of antioxidant enzymes, the peroxiredoxins, which contain one cysteine residue [26]. Pmp20 homologs containing a PTS1 have also been identified in mammals and lower eukaryotes, e.g. *Saccharomyces cerevisiae* (ScPmp20; also designated as Ahp1p or type II thioredoxin peroxidase (TPX)) [27,28], human (HsPmp20), and mouse (MmPmp20) [29]. HsPmp20 and ScPmp20 exhibited antioxidant activity *in vitro*, and both MmPmp20 and ScPmp20 were reported to have TPX activity [30,31]. However, the physiological function of these Pmp20-family proteins, especially within peroxisomes, has been unclear because of their bimodal distribution between peroxisomes and mitochondria.

ChPmp20 is specifically induced during growth of cells on methanol and is exclusively localized within peroxisomes [32,33]. ChPmp20 showed glutathione peroxidase activity, reactive against alkyl hydroperoxides and H2O2 [34]. Catalytic
activity and dimerization of CbPmp20 depended on the only cysteine residue corresponding to Cys53. The PMP20 deletion strain (pmp20Δ) showed reduced growth properties on methanol as a carbon and energy source. Interestingly, the growth defect of the pmp20Δ strain was more severe than that of the cta1Δ strain [20]. During incubation of these strains in methanol media, the CAT deletion strain (cta1Δ) strain accumulated H2O2, while the pmp20Δ strain did not. Therefore, the main function of Pmp20 is thought to degrade ROS generated at the peroxisomal membrane surface, e.g. lipid hydroperoxides, rather than to degrade H2O2. In addition, we detected a physiological level of reduced glutathione in purified peroxisomal fractions of C. boidinii [34]. These results have revealed physiological significances of CbPmp20 as an antioxidant enzyme within peroxisomes rich in ROS.

3.3. Formaldehyde assimilation and dissimilation

Dihydroxyacetone synthase (DHAS) (EC 2.2.1.3) catalyzes the first reaction in the assimilation pathway by fixing formaldehyde to xylulose-5-phosphate. The native enzyme is a homodimer with a molecular weight of approximately 155 kDa. TPP and Mg2+ serve as cofactors for DHAS. The enzyme is a special transketolase which catalyses the glycolaldehyde transfer from xylulose-5-P as a donor to the acceptor molecule formaldehyde by way of a ping-pong mechanism [35].

The formaldehyde produced inside peroxisomes is immediately donated to and fixed by DHAS when sufficient xylulose-5-phosphate is present within the organelle. If not, formaldehyde diffuses to the cytosol, where it is further oxidized via the dissimilatory pathway to CO2 through glutathione-dependent formaldehyde dehydrogenase (FDH) (EC 1.2.1.1), S-formyl glutathione hydrolase (FGH) (EC 3.1.2.12) and formate dehydrogenase (FDH) (EC 1.2.1.2) [9].

The presence of DHAS in close association with AO (see Fig. 1F) is important in regulating the flux of formaldehyde, generated from methanol, over the assimilatory and dissimilatory pathway (see also Section 4). For formaldehyde assimilation sufficient amounts of xylulose-5-P have to be present inside peroxisomes. The generation of xylulose-5-P via the xylulose-5-P cycle involves the ATP dependent phosphorylation of dihydroxyacetone by dihydroxyacetone kinase in the cytosol (see Fig. 2). Import of xylulose-5-P into the organelle is most likely an active transport process, although the transporter is still unknown. Hence, when the energy status of the cells is high, the rate of supply of xylulose-5-P to peroxisome located DHAS is stimulated, which allows the flux of formaldehyde into the direction of assimilation to be increased. In the opposite case – at energy limitation conditions – the supply of xylulose-5-P to DHAS will be reduced, leading to diffusion of formaldehyde into the cytosol. There, it is trapped by reduced glutathione (GSH) that is present at high concentrations, thus rendering formaldehyde a suitable substrate for formaldehyde dehydrogenase [9]. Dissimilation of formaldehyde results in the generation of NADH which, via mitochondrial oxidative phosphorylation, leads to the production of ATP and hence an enhanced energy status of the cell. The increased ATP levels will stimulate the formation of xylulose-5-P and import of this compound into peroxisomes, causing that the assimilatory pathway will be enhanced (Fig. 2A). Hence, the proper partitioning of formaldehyde over assimilatory and dissimilatory pathways is fully controlled by the energy status of the cells.

4. The role of peroxisomes in C1 metabolism: WT versus pex mutants

As indicated above, compartmentalization of AO and DHAS are crucial to allow energy generation from formaldehyde oxidation to CO2 (see Fig. 2). However, also the peroxisomal location of CAT is crucial to allow growth of yeast cells on methanol. This was not the anticipated result as H. polymorpha is capable of coping with significant amounts of exogenously added hydrogen peroxide (at rates up to 8 mmol H2O2 g of cells⁻¹ h⁻¹) [19]. These data demonstrated that peroxisomal CAT was not a key player in decomposing exogenous H2O2 which is instead most likely metabolized via mitochondrial peroxidase activities [19].

The need to decompose H2O2 at its site of generation in the peroxisome to allow growth of H. polymorpha cells on methanol as sole carbon source became clear in studies of van der Klei et al. [8] on peroxisome-deficient (pex) mutants of the organism. A general overview of methanol metabolism in H. polymorpha pex mutant cells [8] is presented in Fig. 2B.

Obviously, in H. polymorpha pex mutants the peroxisome-borne enzymes of methanol metabolism (AO, CAT and DHAS) are present in the cytosol. However, the cytosolic location of CAT leads to major energetic disadvantages. First, in pex mutants, the significance of CAT in H2O2 metabolism is of minor importance because of the relatively low affinity of the enzyme for the H2O2 substrate relative to e.g. cytochrome c peroxidase (CCP). However, CCP mediated H2O2 metabolism is an energy-(NADH) dependent process. Consistent with this, H2O2 metabolism in cells in which CAT activity is inhibited leads to a significant reduction in overall cell yield [9,19]. A second effect that will negatively influence the negative energy balance is the non-enzymatic oxidation of glutathione (GSH) by H2O2. This has a 2-fold negative effect namely (i) the amount of GSH available to condense with formaldehyde to form the formaldehyde dehydrogenase substrate will lead to a reduction in dissimilation (and thus energy generation) and (ii) the presence of insufficient amounts of GSH will cause increasing levels of free formaldehyde in the cytosol. However, at the cytosolic pH (pH≈7.2) free formaldehyde will be hydrated to form methylene glycol, which is a suitable substrate for AO and will be oxidized to form formate. Hence, in this scenario only one reduction equivalent will be formed from the oxidation of methanol into CO2 relative to the two NADH molecules that are formed at WT conditions. Hence, at GSH limiting conditions the net energy balance may become negative as only one reduction equivalent is formed from methanol dissimilation whereas two are required for oxidation of H2O2 by CCP and oxidation/reduction of GSH (see Fig. 2B). Obviously, cells cannot grow at such conditions.

In conclusion, intact peroxisomes are crucial to support growth of cells on methanol as sole source of carbon and energy.
Even relative low amounts of cytosolic AO activities will interfere with methanol growth. The function of the organelles during methylotrophic growth is to allow (i) proper partitioning of formaldehyde generated from methanol over the assimilatory and dissimilatory pathways and (ii) to ascertain $\text{H}_2\text{O}_2$ scavenging at the sites where it is produced to prevent metabolism of this compound by energy consuming processes [8].

5. The roles of $S$-formylglutathione hydrolase

$S$-formylglutathione hydrolase (FGH, EC 3.1.2.12) hydrolyzes $S$-formylglutathione to formate and glutathione (GSH) (Fig. 2A). Together with formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH), FGH is involved in the glutathione-dependent formaldehyde oxidation pathway [36]. FGH is speculated to have two important functions in yeast C1-metabolism namely as a component of the formaldehyde oxidation pathway and as the reaction that supplies GSH to the intracellular pool. To study the physiological role of FGH in methylotrophic yeast, a gene encoding FGH, $FGH_1$, was cloned from $C.\ boidinii$ and functionally characterized [37].

The predicted amino acid sequence of $C.\ boidinii$ FGH1 showed more than 60% similarity to those of FGHs from $\text{Paracoccus} \denitrificans$ and $S.\ cerevisiae$, and human esterase D. Interestingly, $C.\ boidinii$ FGH contained a C-terminal tripeptide, SKL, which is a type I peroxisome targeting signal. By means of subcellular fractionation experiments and observation of GFP-tagged FGHs, FGH has been shown to exhibit a bimodal distribution between the cytosol and peroxisomes. However, the C-terminal SKL tripeptide is not essential for its physiological function. The formaldehyde oxidation pathway has been thought to exist exclusively in the cytosol because two other enzymes, FLD and FDH, are localized in the cytosol [36]. One possible explanation for the peroxisomal existence of FGH is as follows: a portion of $S$-formylglutathione, a product of the FLD-catalyzed reaction and a substrate for FGH, is transported from the cytosol to peroxisomes and FGH may release GSH within peroxisomes. GSH in peroxisomes is required for the glutathione peroxidase activity of CbPmp20, which detoxifies reactive oxygen species within peroxisomes as mentioned above.

The $fgh1$ strain was not able to grow on methanol in batch cultures, but still able to grow on methanol as a carbon source in methanol-limited chemostat cultures at low dilution rates ($D<0.05\text{ h}^{-1}$). These results suggested that FGH is not essential but necessary for optimal growth on methanol. In the $fgh1$ strain, GSH is not released from $S$-formylglutathione, which would lead to further inefficiency as to formaldehyde oxidation and energy generation. In wild-type cells, GSH released from $S$-formylglutathione would be transported to peroxisomes where GSH could react with formaldehyde to enter into another cycle of the formaldehyde oxidation pathway or could be used as a reducing agent for CbPmp20 antioxidant activity. In fact, the $fgh1$ cells (0.125 mM/mg protein) had a lower concentration of GSH than the $fdh1$ cells (0.309 mM/mg protein) [37]. Therefore, besides being a component of the formaldehyde oxidation pathway, FGH seems to play an important role in the regeneration of GSH.

6. Sorting and assembly of peroxisomal C1 enzymes

6.1. Targeting of C1 enzymes involves the PTS1 receptor $\text{Pex5p}$

AO, CAT and DHAS are all imported into the peroxisomal matrix via the Pex5p dependent peroxisomal targeting signal 1 (PTS1) protein import pathway. All three proteins contain conserved variants of the typical PTS1 sequence –SKL (Table 1). Studies in $H.\ polymorpha$ indicated that both DHAS and CAT can be imported into peroxisomes in the fully assembled oligomeric form. AO however follows an alternative pathway and is imported into peroxisomes as FAD containing AO monomers [38].

6.2. Sorting of AO involves a special Pex5p dependent pathway

Although import of AO is dependent on the PTS1 receptor Pex5p, this PTS1 of AO is redundant for its import into peroxisomes. Detailed studies revealed that sorting of AO molecules occurs via an exceptional pathway [16] (for an extensive review on this topic the reader is also referred to chapter 3.1.6 of this issue of BBA). In short, our current model of the AO biosynthetic pathway predicts that binding of the co-factor FAD to newly synthesized AO monomers in the cytosol is the first step in the AO sorting and assembly pathway. Surprisingly, this process is dependent on the presence of the cytosolic protein pyruvate carboxylase protein (Pyc1p). Mutagenesis studies revealed that not the enzyme activity of Pyc1p but another function of this protein is required for FAD binding to AO monomers. Next, the FAD-containing AO monomers are recognized by Pex5p. This interaction is independent of the PTS1 of AO and involves the N-terminal domain of Pex5p, instead of the C-terminal TPR domains which are normally involved in PTS1 binding. Upon import of the Pex5p/AO complex in the peroxisomal matrix the AO monomers dissociate from their receptor and assemble into enzymatic active AO octamers.

This highly specialized AO import pathway may exist because of two major physiological reasons. First, as FAD binding is a pre-requisite for recognition of AO by its receptor Pex5p, this mechanism prevent the import and accumulation of FAD-lacking AO molecules inside peroxisomes. Secondly, import of inactive AO monomers reduces the risk of the release of enzymatic active AO octamers in the cytosol, which severely

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Yeast species</th>
<th>C-terminal residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>$AOD1$</td>
<td>$C.\ boidinii$</td>
<td>-ARY</td>
</tr>
<tr>
<td>AO</td>
<td>$MOD1$</td>
<td>$P.\ methanolica$</td>
<td>-ARF</td>
</tr>
<tr>
<td>AO</td>
<td>$MOD2$</td>
<td>$P.\ methanolica$</td>
<td>-GRF</td>
</tr>
<tr>
<td>AO</td>
<td>$AOX$</td>
<td>$H.\ polymorpha$</td>
<td>-AKL</td>
</tr>
<tr>
<td>AO</td>
<td>$AOX1$</td>
<td>$P.\ pastoris$</td>
<td>-ARF</td>
</tr>
<tr>
<td>AO</td>
<td>$AOX2$</td>
<td>$P.\ pastoris$</td>
<td>-ARF</td>
</tr>
<tr>
<td>CAT</td>
<td>$CTA1$</td>
<td>$C.\ boidinii$</td>
<td>-NKF</td>
</tr>
<tr>
<td>CAT</td>
<td>$CAT$</td>
<td>$H.\ polymorpha$</td>
<td>-SKI</td>
</tr>
<tr>
<td>DHAS</td>
<td>$DAS1$</td>
<td>$C.\ boidinii$</td>
<td>-NKL</td>
</tr>
<tr>
<td>DHAS</td>
<td>$DAS4$</td>
<td>$H.\ polymorpha$</td>
<td>-NKL</td>
</tr>
</tbody>
</table>
hampers growth of cells on methanol (see Section 4). This is less important for other enzymes like CAT and DHAS, which are imported as assembled, enzymatic active oligomeric proteins.

6.3. Biogenesis of DHAS and the role of Pmp47 in DHAS-folding

The genes coding for DHAS were cloned from H. polymorpha and C. boidinii [35,39]. The deduced amino acid sequences of DHAS from these two strains showed similarities to each other, contained transketolase signatures, a possible TPP binding domain and C-terminal PTS1 sequences (Table 1).

A peroxisomal integral membrane protein, Pmp47, of C. boidinii is involved in the formation of active DHAS [40]. In the PMP47 deletion strain (pmp47Δ), DHAS protein was observed to aggregate in the cytosol as an inclusion body. Pmp47 belongs to a family of mitochondrial solute transporters (e.g. ATP/ADP exchanger), and is the only known peroxisomal member of this family [41]. Pmp47 is also involved in medium-chain fatty acid metabolism [42]. The C. boidinii pmp47Δ strain could grow on long-chain fatty acids including palmitate, myristate and oleate but not on laureate. These results suggested that Pmp47 is involved in the transport of a small molecule (possibly ATP) required in the conversion of laureate to its CoA form in peroxisomes by laureate-CoA synthetase (Faa2p). Therefore, ATP transport by Pmp47 seems to be necessary for the folding or transport process of DHAS.

Evidence that Pmp47 is indeed a peroxisomal ATP transporter came from studies on Ant1p (YPR128cp), a Pmp47 homologue from S. cerevisiae [43,33]. Ant1p was essential for growth on medium-chain fatty acids as the sole carbon source. Van Roermund et al. [43] showed that the ATP dependent peroxisomal luciferase activity was strongly reduced in cells lacking Ant1p (ypr128c cells) compared to wild-type controls. Furthermore, Palmieri et al. [44] demonstrated specific transport of adenine nucleotides upon reconstitution of purified Ant1p into liposomes. Remarkably, both the substrate and inhibitor specificity differed from those of the mitochondrial ADP/ATP transporter. Thus, Pmp47 and its homologs probably transport cytosolic ATP into the peroxisomal lumen in exchange for AMP.

7. Regulation of C1 metabolism

7.1. Regulation of the synthesis of C1 enzymes and peroxins

Most of the key enzymes of methanol metabolism (AO, DHAS, FLD, FDH) are present at high levels during growth of cells on methanol, whereas these C1 enzymes are virtually absent in cell growing exponentially on glucose or ethanol. Expression of none of these enzymes is dependent on the presence of methanol in the medium [10,11,33,45]. For instance, during growth of H. polymorpha cells on media containing glycerol AO levels as high as 60% of the values detected in methanol-grown cells can be obtained. In H. polymorpha derepression also occurs when the levels of glucose become very low, for instance in early stationary glucose batch cultures. Derepression of C1 genes has also been observed in other methylotrophic yeast species, however not to the same extent as in H. polymorpha.

In all methylotrophic yeast glucose and ethanol strongly repress the expression of C1 genes. For other substrates (like glycerol or dihydroxyacetone) the extend of repression depends on the yeast species, whereas use of methanol invariably results in induction.

In addition to the genes encoding C1 enzymes also several PEX genes are induced by methanol [33]. However, expression of PEX genes is not fully repressed by glucose or ethanol and their induction by methanol is relatively weak as compared to the genes encoding C1 enzymes.

Methylotrophic yeast species have a cytosolic formaldehyde-detoxyification pathway [9]. However, since the generation of formaldehyde takes place within peroxisomes, mechanisms for the prevention of a sudden increase in the concentration of formaldehyde in peroxisomes are likely to exist. In this respect, one interesting observation was that induction of DHAS preceded that of AO during the early stages of methanol-induction in C. boidinii [40]. If AO was induced strongly by methanol at an earlier stage than DHAS, a large amount of formaldehyde would accumulate in the peroxisomes. Indeed, the growth of the DHAS deletion strain (das1Δ) strain was strongly inhibited in medium containing both methanol and glycerol where formaldehyde generated by AO could not be metabolized via a dissimilation pathway [35]. This timely fashioned induction of AO and DHAS helps to minimize the toxicity of formaldehyde in the peroxisome [9].

7.2. Regulation of genes encoding AO

Regulation of AOX genes in methylotrophic yeast has been the topic of extensive research. H. polymorpha contains a single gene encoding AO, whereas P. pastoris and P. methanolica harbor two genes, which are regulated differently.

The H. polymorpha AOX promoter (P_{AOX}) contains two upstream activation sequences, UAS1 and UAS2, as well as one upstream repressing sequence, URS1. As indicated above, H. polymorpha AOX is strongly repressed by glucose and ethanol. In the presence of these carbon sources the AOX gene is organized in nucleosomes and hence unavailable for transcription [46]. At derepressing or inducing conditions, the P_{AOX} becomes accessible for the transcription machinery due to the action of a chromatin remodelling complex. Recently, we showed that H. polymorpha Swi1p and Snf2p are components of this complex [47]. In H. polymorpha derepression of the AOX gene already occurs at very low glucose concentrations (for instance in early stationary growth phase of glucose cultures) and also during growth of cells on carbon sources like dihydroxyacetone or glycerol [48]. Induction by methanol results in the highest AO levels, which can reach up to 30% of total cellular protein in methanol-limited chemostat cultures at low dilution rates [10,49].

H. polymorpha MPP1 (methylotrophic peroxisomal protein regulator 1) encodes a transcription factor that is important for growth of cells on methanol [50]. Mpp1p belongs to a family of
zinc cluster proteins that is exclusively present in fungi and contains well conserved DNA binding domains. Deletion of MPP1 completely abolishes the capacity of *H. polymorpha* cells to grow on methanol but has no effect on growth on other carbon sources (glucose, ethanol, glycerol, dihydroxyacetone). Hence, this transcription factor is highly specific for methylo-trophic growth. In *H. polymorpha mpp1* cells the level of AO protein is strongly reduced, whereas DHAS protein is below the level of detection. In addition, the levels of several peroxins are strongly reduced in *mpp1* cells, resulting in a defect in peroxi-some proliferation.

In addition to the transcription factor Mpp1p, several proteins involved in repression of the *H. polymorpha* P\textsubscript{AOX} have been identified. These include a hexose transporter [51], glucokinase and hexokinase [52–54]. Also several mutants have been described that are insensitive to catabolite repression, but the corresponding genes have not yet been identified. However, the number of mutants isolated strongly suggests that several additional proteins exist that play a role in P\textsubscript{AOX} repression.

*P. methanolica* possesses two genes encoding AO, *MOD1* (AUG1) and *MOD2* (AUG2). Expression of both genes is induced by methanol. Native gel electrophoresis of cell-free extract prepared from methanol-grown *P. methanolica* cells revealed nine protein bands that showed AO enzyme activity. These AO isozymes are the result of oligomerization of different ratios of Mod1p and Mod2p subunits into active octamers [55,56]. A strain expressing only *MOD2* shows a severe growth defect in media containing low concentrations of methanol (0.1%), but growth is restored when the concentration of methanol is increased (up to 3%). *MOD2* is not expressed under derepression conditions (0% methanol), but increases when increasing methanol concentrations are used for induction. In contrast, expression of *MOD1* does occur under derepression conditions and does not change with increasing methanol concentrations. Therefore, the ratio of Mod2p and Mod1p subunits in active AO octamers is mainly controlled by variations in the levels of Mod2p subunits. Since Mod2p was shown to have 10-fold lower affinity for methanol compared to Mod1p, an increase in the Mod2p/Mod1p ratio is a mode for the cells to adapt to high concentrations of methanol [57].

Heterologous gene expression driven by the *MOD1* and *MOD2* promoters was observed to be strong and tightly regulated by the carbon source at the transcriptional level [58]. *MOD1* expression was optimal at low methanol concentrations, whereas *MOD2* was predominantly expressed at high methanol and high oxygen concentrations. Based on these results, both promoters are useful tools for controlling heterologous gene expression in *P. methanolica*. In particular, it should be possible to differentially control the production phases of two hetero-logous proteins using *MOD1* and *MOD2* promoters in the same host cell [59].

*P. pastoris* also contains two genes encoding AO, which are designated *AOX1* and *AOX2* and show very strong homology in the coding region [60]. In *P. pastoris* cells AO protein is invariably absent in glucose or glycerol grown cells. In this respect this yeast significantly differs from *H. polymorpha*. *P. pastoris* *AOX2* is expressed at significantly lower levels than *AOX1*, which is strongly induced by methanol. As a result deletion of *AOX2* has only a minor effect on growth of cells on methanol. The large difference in regulation of both genes is also reflected by the observation that there is no similarity between the promoter regions of both *AOX* genes [60].

Recently, a gene was identified that is involved in regulating C1 enzymes in *P. pastoris*. This gene, designated *MXR1* (methanol expression regulator 1), encodes a DNA binding transcription factor which contains two DNA-binding zinc fingers [61]. Cells of a constructed *MXR1* deletion strain (*mxr1Δ*) fully lost the capacity to grow on methanol. Also, growth on oleic acid, another carbon source that requires the function of peroxisomes for its metabolism, was fully impaired. Cells of the *mxr1Δ* strain also showed retarded growth on other carbon sources, but this defect was less severe as relative to that of methanol and oleic acid utilization. In *mxr1Δ* cells the levels of AO and DHAS as well as enzymes of the dissimilation pathway and all tested peroxins were strongly reduced.

**8. Concluding remarks**

Utilization of C1 compounds by eukaryote microorganisms is not a widespread property and is confined to methanol utilization. Surprisingly, eukaryote organisms that can grow on related compounds, e.g. methylamine, as sole carbon source have not been detected. This is somewhat surprising as the metabolic intermediate of this compound is formaldehyde, which is similar to the first oxidation product of methanol.

Methylamine can be used as nitrogen source and is oxidized by a peroxisomal amine oxidase. However, the regulation of amine oxidase synthesis prevents utilization of this compound as nitrogen source and allows the use of methylamine to just satisfy the need for cellular nitrogen [62].

Relative to prokaryotes that can use various C1 compounds for growth the efficiency of methanol metabolism by yeast is poor. The paradox is that this is related to the mode of methanol utilization that is adopted by eukaryotes. Where compartmentalization is thought to be beneficial for specific pathways and indeed does promote methanol utilization in yeast – i.e. to allow proper partitioning of formaldehyde fluxes over carbon fixation and energy generation (see Section 4) – the immediate disadvantage is that oxidation of methanol by AO does not generate energy. An additional disadvantage is that AO activity in the cytosol and an corresponding energetically disadvantageous– H\textsubscript{2}O\textsubscript{2} metabolism in the cytosol (Section 4) strongly interferes growth of cells on methanol. To circumvent this, a specific AO biosynthetic pathway has evolved during evolution. Normally, matrix components of peroxisomes are assembled in the cytosol and imported in their mature form [16,38,63]. AO protein, however, is imported as monomers. These monomers accept their FAD co-factor in the cytosol, a process that requires the function of the protein pyruvate carboxylase [64] and are assembled into the active enzyme in the organelle lumen.

This unexpected complexity may add to the understanding why so relatively few eukaryotes have adopted the capacity to grow on methanol.
Acknowledgements

Y.S. is founded by a Grant-in-Aid for Scientific Research on Priority Areas 399 and 504, the COE program from the Ministry of Education, Science, Sports, and Culture of Japan, and the National Institute for Basic Biology Cooperative Research Program.

We would like to thank Klaas Sjollema, Wieb Meijer and Richard Baerends for the preparation of the figures and table.

References


