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Published in:
Biochimica et Biophysica Acta (BBA) - Molecular Cell Research

DOI:
[10.1016/j.bbamcr.2006.07.016](https://doi.org/10.1016/j.bbamcr.2006.07.016)

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

Publication date:
2006

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Klei, I. J. V. D., Yurimoto, H., Sakai, Y., & Veenhuis, M. (2006). The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1763(12), 1453 - 1462. <https://doi.org/10.1016/j.bbamcr.2006.07.016>

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Review

The significance of peroxisomes in methanol metabolism in methylotrophic yeast

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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.

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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 biphosphate 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 *Biochemistry of Methylotrophs* (Anthony, 1982 [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 [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

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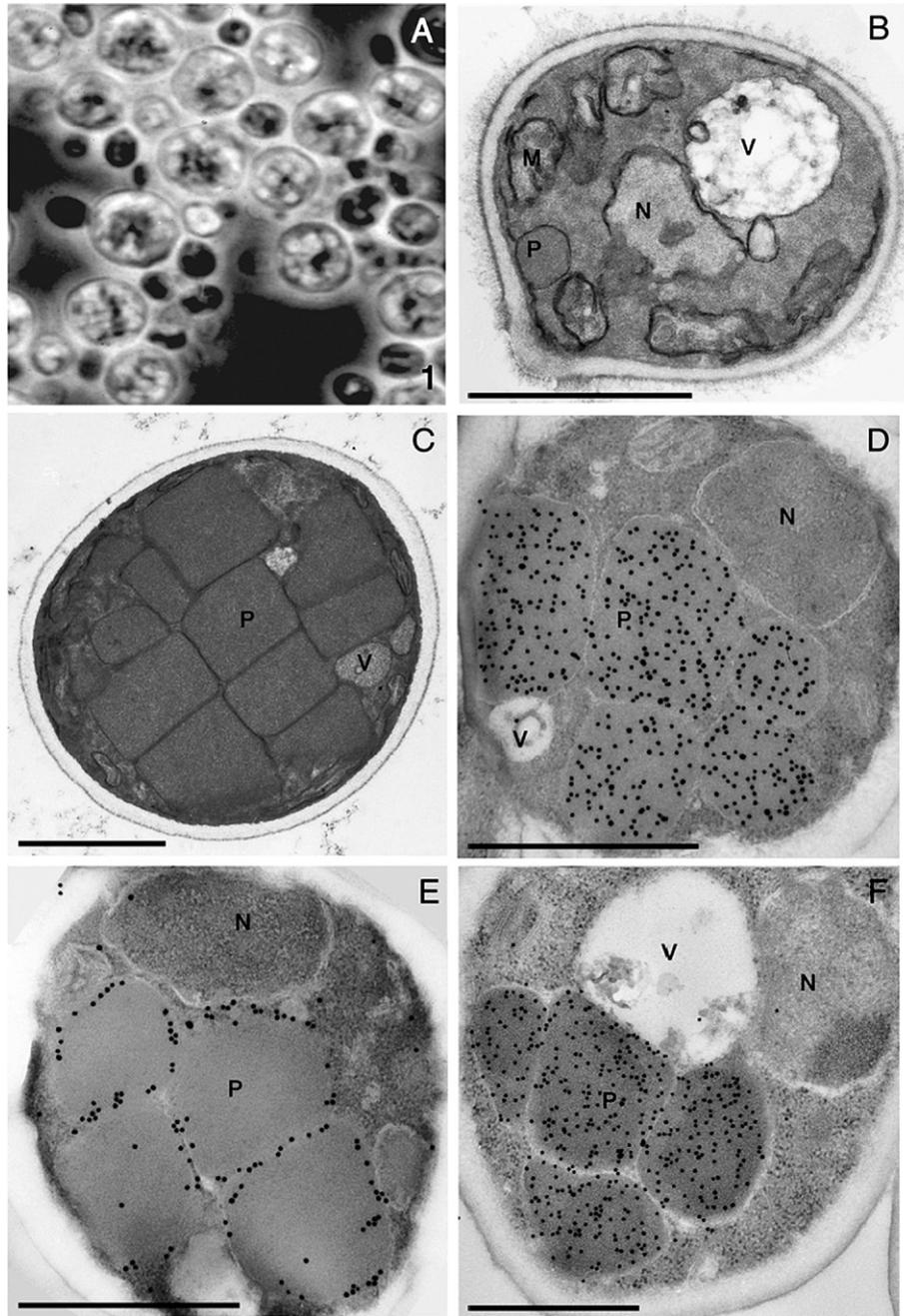


Fig. 1. (A) shows a light micrograph of *H. polymorpha* cells that are grown in a methanol-limited chemostat culture. In the cells the cuboid inclusions that represent peroxisomes are evident. (B) shows an electron micrograph of the same organism, grown in batch culture on glucose. These cells contain only a single small peroxisome. (C) shows the morphology of peroxisomes in methanol-limited grown *H. polymorpha*. (D–F) Immunocytochemical localization of AO (D), CAT (E) and DHAS protein (F) in peroxisomes of cells grown in batch cultures on methanol. Note that DHAS is randomly present over the organelle whereas catalase is predominantly located at the edges of the organelle. For immunocytochemistry specific polyclonal antibodies against the three indicated proteins are used. M—mitochondrion; N—nucleus; P—peroxisome; V—vacuole. The bar represents 1 μm .

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 CO_2 (formaldehyde dehydrogenase, *S*-formylglutathione hydrolase, and formate

2. Methylotrophic yeast species

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

Most methylotrophic yeast strains have first been isolated from samples containing decaying plant material. For instance, *P. pastoris* 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 methylotrophic yeast, because methanol is released during hydrolysis of pectin, a major plant constituent [12].

The massive proliferation of peroxisomes in methylotrophic 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. Methylotrophic 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, 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 methylotrophic 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 methylotrophic 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 ($\beta\alpha\beta$), 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 methylotrophic 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 (CbPmp20), 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.

CbPmp20 is specifically induced during growth of cells on methanol and is exclusively localized within peroxisomes [32,33]. CbPmp20 showed glutathione peroxidase activity, reactive against alkyl hydroperoxides and H_2O_2 [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 glycoaldehyde 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 (FLD) (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 H₂O₂ 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, *FGH1*, was cloned from *C. boidinii* and functionally characterized [37].

The predicted amino acid sequence of CbFGH1 showed more than 60% similarity to those of FGHS from *Paracoccus denitrificans* and *S. cerevisiae*, and human esterase D. Interestingly, CbFGH 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 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 1
PTS1 sequences of the peroxisomal key C1 enzymes in yeast

Protein	Gene	Yeast species	C-terminal residues
AO	<i>AOD1</i>	<i>C. boidinii</i>	-ARY
AO	<i>MOD1</i>	<i>P. methanolica</i>	-ARF
AO	<i>MOD2</i>	<i>P. methanolica</i>	-GRF
AO	<i>AOX</i>	<i>H. polymorpha</i>	-AKL
AO	<i>AOX1</i>	<i>P. pastoris</i>	-ARF
AO	<i>AOX2</i>	<i>P. pastoris</i>	-ARF
CAT	<i>CTA1</i>	<i>C. boidinii</i>	-NKF
CAT	<i>CAT</i>	<i>H. polymorpha</i>	-SKI
DHAS	<i>DAS1</i>	<i>C. boidinii</i>	-NHL
DHAS	<i>DAS</i>	<i>H. polymorpha</i>	-NKL

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 extent 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-detoxification 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 MPPI (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 methylotrophic 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 peroxisome proliferation.

In addition to the transcription factor Mpp1p, several proteins involved in repression of the *H. polymorpha* P_{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_{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 heterologous 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_2O_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 funded 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 Sjollem, Wieb Meijer and Richard Baerends for preparation of the figures and table.

References

- [1] C. Anthony, The biochemistry of methylotrophs, Academic Press, London, United Kingdom, 1982.
- [2] H. van den Bosch, R.B. Schutgens, R.J. Wanders, J.M. Tager, Biochemistry of peroxisomes, *Annu. Rev. Biochem.* 61 (1992) 157–197.
- [3] P.E. Purdue, P.B. Lazarow, Peroxisome biogenesis, *Annu. Rev. Cell Dev. Biol.* 17 (2001) 701–752.
- [4] M. Veenhuis, W. Harder, Microbodies in yeasts: structure, function and biogenesis, *Microbiol. Sci.* 5 (1988) 347–351.
- [5] M. Veenhuis, F.A. Salomons, I.J. van Der Klei, Peroxisome biogenesis and degradation in yeast: a structure/function analysis, *Microsc. Res. Tech.* 51 (2000) 584–600.
- [6] J.M. Cregg, I.J. van der Klei, G.J. Sulter, M. Veenhuis, W. Harder, Peroxisome-deficient mutants of *Hansenula polymorpha*, *Yeast* 6 (1990) 87–97.
- [7] H. Liu, X. Tan, M. Veenhuis, D. McCollum, J.M. Cregg, An efficient screen for peroxisome-deficient mutants of *Pichia pastoris*, *J. Bacteriol.* 174 (1992) 4943–4951.
- [8] I.J. van der Klei, W. Harder, M. Veenhuis, Methanol metabolism in a peroxisome-deficient mutant of *Hansenula polymorpha*: a physiological study, *Arch. Microbiol.* 156 (1991) 15–23.
- [9] H. Yurimoto, N. Kato, Y. Sakai, Assimilation, dissimilation, and detoxification of formaldehyde, a central metabolic intermediate of methylotrophic metabolism, *Chem. Rec.* 5 (2005) 367–375.
- [10] J.P. van Dijken, M. Veenhuis, N.J. Kreger-van Rij, W. Harder, Microbodies in methanol-assimilating yeasts, *Arch. Microbiol.* 102 (1975) 41–44.
- [11] S. Fukui, A. Tanaka, S. Kawamoto, S. Yasuhara, Y. Teranishi, M. Osumi, Ultrastructure of methanol-utilizing yeast cells: appearance of microbodies in relation to high catalase activity, *J. Bacteriol.* 123 (1975) 317–328.
- [12] T. Nakagawa, T. Miyaji, H. Yurimoto, Y. Sakai, N. Kato, N. Tomizuka, A methylotrophic pathway participates in pectin utilization by *Candida boidinii*, *Appl. Environ. Microbiol.* 66 (2000) 4253–4257.
- [13] Y. Sakai, S. Subramani, Environmental response of yeast peroxisomes. Aspects of organelle assembly and degradation, *Cell Biochem. Biophys.* 32 (2000) 51–61.
- [14] K.N. Faber, Y. Elgersma, J.A. Heyman, A. Koller, G.H. Luers, W.M. Nuttley, S.R. Terlecky, T.J. Wenzel, S. Subramani, Use of *Pichia pastoris* as a model eukaryotic system. Peroxisome biogenesis, *Methods Mol. Biol.* 103 (1998) 121–147.
- [15] G. Gellissen, G. Kunze, C. Gaillardin, J.M. Cregg, E. Berardi, M. Veenhuis, I.J. van der Klei, New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adenivorans* and *Yarrowia lipolytica*—A comparison, *FEMS Yeast Res.* 5 (2005) 1079–1096.
- [16] P.Z. Ozimek, M. Veenhuis, I.J. van der Klei, Alcohol oxidase: a complex peroxisomal, oligomeric flavoprotein, *FEMS Yeast Res.* 5 (2005) 975–983.
- [17] R. Boteva, A.J. Visser, B. Filippi, G. Vriend, M. Veenhuis, I.J. van der Klei, Conformational transitions accompanying oligomerization of yeast alcohol oxidase, a peroxisomal flavoenzyme, *Biochemistry* 38 (1999) 5034–5044.
- [18] I. Keizer, R. Roggenkamp, W. Harder, M. Veenhuis, Location of catalase in crystalline peroxisomes of methanol-grown *Hansenula polymorpha*, *FEMS Microbiol. Lett.* 72 (1992) 7–11.
- [19] C. Verduyn, M.L. Giuseppin, W.A. Scheffers, J.P. van Dijken, Hydrogen peroxide metabolism in yeasts, *Appl. Environ. Microbiol.* 54 (1988) 2086–2090.
- [20] H. Horiguchi, H. Yurimoto, T.K. Goh, T. Nakagawa, N. Kato, Y. Sakai, Peroxisomal catalase in the methylotrophic yeast *Candida boidinii*: transport efficiency and metabolic significance, *J. Bacteriol.* 183 (2001) 6372–6383.
- [21] I. Singh, Mammalian peroxisomes: metabolism of oxygen and reactive oxygen species, *Ann. N.Y. Acad. Sci.* 804 (1996) 612–627.
- [22] G.A. Keller, T.G. Warner, K.S. Steimer, R.A. Hallwell, Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 7381–7385.
- [23] G.S. Dhaunsi, S. Gulati, A.K. Singh, J.K. Orak, K. Asayama, I. Singh, Demonstration of Cu–Zn superoxide dismutase in rat liver peroxisomes. Biochemical and immunochemical evidence, *J. Biol. Chem.* 267 (1992) 6870–6873.
- [24] A.K. Singh, G.S. Dhaunsi, M.P. Gupta, J.K. Orak, K. Asayama, I. Singh, Demonstration of glutathione peroxidase in rat liver peroxisomes and its intraorganellar distribution, *Arch. Biochem. Biophys.* 315 (1994) 331–338.
- [25] L.J. Garrard, J.M. Goodman, Two genes encode the major membrane-associated protein of methanol-induced peroxisomes from *Candida boidinii*, *J. Biol. Chem.* 264 (1989) 13929–13937.
- [26] E. Schröder, C.P. Ponting, Evidence that peroxiredoxins are novel members of the thioredoxin fold superfamily, *Protein Sci.* 7 (1998) 2465–2468.
- [27] J. Lee, D. Spector, C. Godon, J. Labarre, M.B. Toledano, A new antioxidant with alkyl hydroperoxide defense properties in yeast, *J. Biol. Chem.* 274 (1999) 4537–4544.
- [28] J.S. Jeong, S.J. Kwon, S.W. Kang, S.G. Rhee, K. Kim, Purification and characterization of a second type thioredoxin peroxidase (type II TPx) from *Saccharomyces cerevisiae*, *Biochemistry* 38 (1999) 776–783.
- [29] H. Yamashita, S. Avraham, S. Jiang, R. London, P.P. Van Veldhoven, S. Subramani, R.A. Rogers, H. Avraham, Characterization of human and murine PMP20 peroxisomal proteins that exhibit antioxidant activity in vitro, *J. Biol. Chem.* 274 (1999) 29897–29904.
- [30] Y. Zhou, K.H. Kok, A.C. Chun, C.M. Wong, H.W. Wu, M.C. Lin, P.C. Fung, H. Kung, D.Y. Jin, Mouse peroxiredoxin V is a thioredoxin peroxidase that inhibits p53-induced apoptosis, *Biochem. Biophys. Res. Commun.* 268 (2000) 921–927.
- [31] L. Verdoucq, F. Vignols, J.P. Jacquot, Y. Chartier, Y. Meyer, *In vivo* characterization of a thioredoxin h target protein defines a new peroxiredoxin family, *J. Biol. Chem.* 274 (1999) 19714–19722.
- [32] J.M. Goodman, S.B. Trapp, H. Hwang, M. Veenhuis, Peroxisomes induced in *Candida boidinii* by methanol, oleic acid and D-alanine vary in metabolic function but share common integral membrane proteins, *J. Cell Sci.* 97 (1990) 193–204.
- [33] Y. Sakai, H. Yurimoto, H. Matsuo, N. Kato, Regulation of peroxisomal proteins and organelle proliferation by multiple carbon sources in the methylotrophic yeast, *Candida boidinii*, *Yeast* 14 (1998) 1175–1187.
- [34] H. Horiguchi, H. Yurimoto, N. Kato, Y. Sakai, Antioxidant system within yeast peroxisome—Biochemical and physiological characterization of CbPmp20 in the methylotrophic yeast *Candida boidinii*, *J. Biol. Chem.* 276 (2001) 14279–14288.
- [35] Y. Sakai, T. Nakagawa, M. Shimase, N. Kato, Regulation and physiological role of the *DAS1* gene, encoding dihydroxyacetone synthase, in the methylotrophic yeast *Candida boidinii*, *J. Bacteriol.* 180 (1998) 5885–5890.
- [36] H. Yurimoto, Y. Sakai, N. Kato, Methanol metabolism, in: G. Gellissen (Ed.), *Hansenula polymorpha*—Biology and Applications, Wiley-VCH, Weinheim, 2002, pp. 61–75.
- [37] H. Yurimoto, B. Lee, T. Yano, Y. Sakai, N. Kato, Physiological role of S-formylglutathione hydrolase in C₁ metabolism of the methylotrophic yeast *Candida boidinii*, *Microbiology* 149 (2003) 1971–1979.
- [38] K.N. Faber, R. van Dijk, I. Keizer-Gunnink, A. Koek, I.J. van der Klei, M. Veenhuis, Import of assembled PTS1 proteins into peroxisomes of the yeast *Hansenula polymorpha*: yes and no! *Biochim. Biophys. Acta* 1591 (2002) 157–162.

- [39] Z. Janowicz, M. Eckart, C. Drewke, R. Roggenkamp, C.P. Hollenberg, J. Maat, A.M. Ledebøer, C. Visser, C.T. Verrips, Cloning and characterization of the *DAS* gene encoding the major methanol assimilatory enzyme from the methylotrophic yeast *Hansenula polymorpha*, Nucl. Acids Res. 13 (1985) 3043–3062.
- [40] Y. Sakai, A. Saiganji, H. Yurimoto, K. Takabe, H. Saiki, N. Kato, The absence of Pmp47, a putative yeast peroxisomal transporter, causes a defect in transport and folding of a specific matrix enzyme, J. Cell Biol. 134 (1996) 37–51.
- [41] M.T. McCammon, J.A. McNew, P.J. Willy, J.M. Goodman, An internal region of the peroxisomal membrane protein PMP47 is essential for sorting to peroxisomes, J. Cell Biol. 124 (1994) 915–925.
- [42] T. Nakagawa, T. Imanaka, M. Morita, K. Ishiguro, H. Yurimoto, A. Yamashita, N. Kato, Y. Sakai, Peroxisomal membrane protein Pmp47 is essential in the metabolism of middle-chain fatty acid in yeast peroxisomes and is associated with peroxisome proliferation, J. Biol. Chem. 275 (2000) 3455–3461.
- [43] C.W. van Roermund, R. Drissen, M. van den Berg, L. Ijlst, E.H. Hettema, H.F. Tabak, H.R. Waterham, R.J.A. Wanders, Identification of a peroxisomal ATP carrier required for medium-chain fatty acid β -oxidation and normal peroxisomal proliferation in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 21 (2001) 4321–4329.
- [44] L. Palmieri, H. Rottensteiner, W. Girzalsky, P. Scarcia, F. Palmieri, R. Erdmann, Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter, EMBO J. 20 (2001) 5049–5059.
- [45] H. Yurimoto, T. Komeda, C.R. Lim, T. Nakagawa, K. Kondo, N. Kato, Y. Sakai, Regulation and evaluation of five methanol-inducible promoters in the methylotrophic yeast *Candida boidinii*, Biochim. Biophys. Acta 1493 (2000) 56–63.
- [46] G. Costanzo, E. di Mauro, R. Negri, G. Pereira, C. Hollenberg, Multiple overlapping positions of nucleosomes with single *in vivo* rotational setting in the *Hansenula polymorpha* RNA polymerase II MOX promoter, J. Biol. Chem. 270 (1995) 11091–11097.
- [47] P. Ozimek, K. Lahtchev, J.A.K.W. Kiel, M. Veenhuis, I.J. van der Klei, *Hansenula polymorpha* Swi1p and Snf2p are essential for methanol utilisation, FEMS Yeast Res. 4 (2004) 673–682.
- [48] R. Roggenkamp, Z. Janowicz, B. Stanikowski, C.P. Hollenberg, Biosynthesis and regulation of the peroxisomal methanol oxidase from the methylotrophic yeast *Hansenula polymorpha*, Mol. Gen. Genet. 194 (1984) 489–493.
- [49] J.P. van Dijken, R. Otto, W. Harder, Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism, Arch. Microbiol. 111 (1976) 137–144.
- [50] A.N. Leao-Helder, A.M. Krikken, I.J. van der Klei, J.A.K.W. Kiel, M. Veenhuis, Transcriptional down-regulation of peroxisome numbers affects selective peroxisome degradation in *Hansenula polymorpha*, J. Biol. Chem. 278 (2003) 40749–40756.
- [51] O.V. Stasyk, O.G. Stasyk, J. Komduur, M. Veenhuis, J.M. Cregg, A.A. Sibiry, A hexose transporter homologue controls glucose repression in the methylotrophic yeast *Hansenula polymorpha*, J. Biol. Chem. 279 (2004) 8116–8125.
- [52] T. Kramarenko, H. Karp, A. Jarviste, T. Alamae, Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants, Folia Microbiol. (Praha) 45 (2000) 521–529.
- [53] S. Laht, H. Karp, P. Kotka, A. Jarviste, T. Alamae, Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*, Gene 296 (2002) 195–203.
- [54] H. Karp, A. Jarviste, T.M. Kriegel, T. Alamae, Cloning and biochemical characterization of hexokinase from the methylotrophic yeast *Hansenula polymorpha*, Curr. Genet. 44 (2003) 268–276.
- [55] T. Nakagawa, H. Mukaiyama, H. Yurimoto, Y. Sakai, N. Kato, Alcohol oxidase hybrid oligomers formed *in vivo* and *in vitro*, Yeast 15 (1999) 1223–1230.
- [56] T. Nakagawa, Y. Sakai, H. Mukaiyama, T. Mizumura, T. Miyaji, H. Yurimoto, N. Kato, N. Tomizuka, Analysis of alcohol oxidase isozymes in gene-disrupted strains of methylotrophic yeast *Pichia methanolica*, J. Biosci. Bioeng. 91 (2001) 225–227.
- [57] T. Nakagawa, T. Mizumura, H. Mukaiyama, T. Miyaji, H. Yurimoto, N. Kato, Y. Sakai, N. Tomizuka, Physiological role of the second alcohol oxidase gene *MOD2* in the methylotrophic growth of *Pichia methanolica*, Yeast 19 (2002) 1067–1073.
- [58] T. Nakagawa, A. Inagaki, T. Ito, S. Fujimura, T. Miyaji, H. Yurimoto, N. Kato, Y. Sakai, N. Tomizuka, Regulation of two distinct alcohol oxidase promoters in the methylotrophic yeast *Pichia methanolica*, Yeast 23 (2006) 15–22.
- [59] C.K. Raymond, T. Bukowski, S.D. Holderman, A.F.T. Ching, E. Vanaja, M.R. Stamm, Development of the methylotrophic yeast, *Pichia methanolica*, for the expression of the 65-kilodalton isoform of human glutamate decarboxylase, Yeast 14 (1998) 11–23.
- [60] J.M. Cregg, K.R. Madden, K.J. Barringer, G.P. Thill, C.A. Stillman, Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*, Mol. Cell. Biol. 9 (1989) 1316–1323.
- [61] G.P. Lin-Cereghino, L. Godfrey, B.J. de la Cruz, S. Johnson, S. Khuongsathiene, I. Tolstorukov, M. Yan, J. Lin-Cereghino, M. Veenhuis, S. Subramani, J.M. Cregg, Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in *Pichia pastoris*, Mol. Cell. Biol. 26 (2006) 883–897.
- [62] K.B. Zwart, W. Harder, Regulation of some alkylated amines in the yeasts *Candida utilis* and *Hansenula polymorpha*, J. Gen. Microbiol. 129 (1983) 3157–3169.
- [63] M.Q. Stewart, R.D. Esposito, J. Gowani, J.M. Goodman, Alcohol oxidase and dihydroxyacetone synthase, the abundant peroxisomal proteins of methylotrophic yeasts, assemble in different cellular compartments, J. Cell Sci. 114 (2001) 2863–2868.
- [64] P.Z. Ozimek, R. van Dijk, K. Latchev, C. Gancedo, D.Y. Wang, I.J. van der Klei, M. Veenhuis, Pyruvate carboxylase is an essential protein in the assembly of yeast peroxisomal oligomeric alcohol oxidase, Mol. Biol. Cell 14 (2003) 786–797.