**Abstract**  
The *Hansenula polymorpha* per6-210 mutant is impaired in respect of growth on methanol (Mut–) and is characterized by aberrant peroxisome formation. The functionally complementing DNA fragment contains two open reading frames. The first encodes dihydroxyacetone kinase (DAK), a cytosolic enzyme essential for formaldehyde assimilation; the second ORF codes for a novel protein (Pak1p). We have demonstrated that per6-210 cells lack DAK activity, causing the Mut– phenotype, and have strongly reduced levels of Pak1p, resulting in peroxisomal defects. Sequence analysis revealed that per6-210 contains a mutation in the 3′ end of the DAK coding region, which overlaps with the promoter region of *PAK1*. Possibly this mutation also negatively affects *PAK1* expression.

**Key words** Peroxisome biogenesis · Methanol metabolism · Yeast

**Introduction**

Peroxisomes are essential for growth of methylotrophic yeasts on methanol as the sole source of carbon and energy. These organelles harbour the key enzymes of methanol metabolism: alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). Other enzymes of the dissimilatory (formaldehyde and formate dehydrogenase) and assimilatory (the xylulose-5-phosphate) pathways are located in the cytosol (reviewed by Veenhuis and Harder 1988). In order to identify the genes essential for the growth of methylotrophic yeasts on methanol, various methanol-utilization-deficient (Mut–) mutants of the yeast *Hansenula polymorpha* have been isolated (De Koning et al. 1987; Cregg et al. 1990). Within these collections different classes of Mut– strains have been discriminated, namely: (1) strains mutated in structural genes encoding peroxisomal or cytosolic enzymes involved in methanol metabolism (Verduyn et al. 1984; de Koning et al. 1987), and (2) *pex* mutants, which are defective in peroxisome biogenesis and/or function (Cregg et al. 1990). In these mutants the Mut– phenotype is due to the mislocation of enzymatically active peroxisomal enzymes to the cytosol (van der Klei et al. 1991).

Here, we describe the *H. polymorpha* per6-210 mutant, which has been identified within a previously described collection of *H. polymorpha* Mut– mutants (Titorenko et al. 1993). We found that this mutant is deficient in an enzyme required for methanol metabolism, but is also disturbed in peroxisome formation. We show that a single mutation in *per6-210* affects the protein products of two adjacent genes. The first encodes the enzyme dihydroxyacetone kinase (DAK), a cytosolic enzyme essential for the assimilation of formaldehyde produced from methanol oxidation. The second ORF, designated *PAK1*, encodes a novel protein. The peroxisomal defect in *per6-210* is most probably due to strongly reduced levels of Pak1p.

**Materials and methods**

*Organisms and growth conditions.* *H. polymorpha* wild-type (CBS 4732), the *per6-210* mutant (Titorenko et al. 1993), NCYC 495 *leu1* (Waterham et al. 1994), NCYC 495 *leu1 ura1* (Waterham et al. 1994), and the constructed disruption strains (see below) were grown...
at 37°C on rich complex medium (YPD) containing 1% yeast extract, 2% peptone and 1% glucose, on mineral media as described (van Dijken et al. 1976), or on YNB without amino acids containing 0.67% Yeast Nitrogen Base (Difco). The carbon sources used were 0.5% glucose, 0.5% ethanol, 0.5% glycerol, 0.5% dihydroxyacetone or 0.5% methanol; as nitrogen sources ammonium sulphate or methylamine (both at 0.2%) were employed. Carbon-limited continuous culturing was carried out as described previously (van Dijken et al. 1976) at a dilution rate of 0.05 h⁻¹. Amino acids and uracil were added to a final concentration of 30 μg/ml. To mineral media the vitamins thiamine (20 μg l⁻¹) and biotin (600 μg l⁻¹) were added, unless indicated otherwise. Escherichia coli strains DH5α (GIBCO-BRL, Gaithersburg, Md.) and C600 (Stratagene, La Jolla, Calif.) were grown at 37°C in LB medium or in minimal M9 medium (Sambrook et al. 1989), supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) when required.

Miscellaneous DNA techniques. Standard recombinant DNA techniques, E. coli transformation and plasmid isolation were performed essentially as described (Sambrook et al. 1989). *H. polymorpha* was transformed by electroporation (Faber et al. 1994).

Cloning and characterization of the complementing genes. *H. polymorpha per6-210* was transformed with a genomic DNA library of *H. polymorpha* (Waterham et al. 1994). A plasmid containing a complementing fragment of approximately 5.2 kb was isolated. DNA fragments comprising 4.4 kb of the insert were subcloned in pBluescript (Stratagene, La Jolla, Calif., USA) and sequenced. Nested deletions were generated using Exonuclease III and S1 nuclease as described (Sambrook et al. 1989). Double-stranded sequencing was performed by the dideoxy chain-termination method (Sanger et al. 1977). For analysis of the DNA and amino-acid sequences the PC-GENE™-program, release 6.70 (IntelliGenetics Inc.), was employed. The BLASTN, BLASTP and TBLASTN algorithms (Altschul et al. 1990) were used to screen the GenBank database and employed. The BLASTN, BLASTP and TBLASTN algorithms (Altschul et al. 1990) were used to screen the GenBank database and the *Saccharomyces Genome Database* (SGD, Stanford University, Calif.) for proteins having similarity to the predicted gene products. In order to identify the mutation in per6-210 a DNA fragment, including the 3’ end of ORF1, where the mutation was expected to be located (see Results section), was sequenced. For this purpose a PCR reaction was performed using a proof-reading enzyme and chromosomal DNA isolated from the per6-210 mutant. The following primers were used: 5’-CTC GTC GGA CGA GGT TGT GC-3’ (nucleotides 1269–1288) and 5’-CGG TGA TGG TCT TCA CGT CC-3’ (nucleotides 2480–2499 in GenBank accession no. AF061946). From the resulting PCR product a 1.1-kb XhoI-NcoI fragment (nucleotides 1321–2442, comprising the 3’ end of DAK and the 5’ end of PAK1) was cloned into pBluescript II SK⁺ (Stratagene) and sequenced.

Gene disruptions. For the disruption of the *H. polymorpha* DAK and PAK1 genes we constructed the integration plasmids pMH2 and pMH4, containing internal fragments of either one of the genes. Plasmid pMH2 was constructed as follows. First a 770-bp TthI (blunted)-PstI fragment of PAK1 (nucleotides 2669–3441 in GenBank accession no. AF061946) was cloned into *Hinfl*-digested pBluescript II SK⁺, resulting in plasmid pMH1. Subsequently a 2.2-kb (EcoRI-BamHI) (blunted) fragment containing the *Candida albicans* LEU2 gene (obtained from Dr. E. Berardi, University of Ancona, Ancona, Italy) was inserted in the Smal site of pMH1. Plasmid pMH4 was constructed in a similar way. First a 650-bp PvuII-Apal internal fragment from the DAK gene (nt 1357–2003) was ligated between the EcoRV and Apal sites of pBluescript II SK⁺ resulting in plasmid pMH3. Subsequently, a 2.2-kb EcoRI-BamHI fragment, carrying the *C. albicans* LEU2 gene, was ligated between the EcoRI and BamHI sites of pMH3. Plasmids pMH2 and pMH4 were digested with EcoRI and MluI in the PAK1 and DAK regions, respectively, and integrated in the *H. polymorpha* NCYC 495 *leu1.1 ura1* genome (Faber et al. 1994). A DAK Δpak1 double mutant, in which both the 3’ end of DAK (coding for amino acids 587–609) and the 5’ end of PAK1 (coding for amino acids 1–167) were deleted, was constructed by directed integration using a DAK-PAK1 PvuII fragment (nt 1357–4422) in which the BamHI-EcoRI portion (nt 2208–2828) was replaced by a 2.2-kb EcoRI-BamHI fragment containing the *C. albicans* LEU2 gene (Fig. 1). In all cases leucine prototrophic transformants were checked for the proper insertion into the *H. polymorpha* genome by Southern analysis, using the ECL gene detection system (Amersham International, Amersham, UK) as recommended by the supplier (data not shown).

Construction of a Pak1p over-producing strain. Overexpression of PAK1 was achieved as follows. First, a BamHI site was introduced upstream of the first start codon of PAK1 by PCR (5’ primer: TGT GGA TCC AAA ATG GAG GCC TTT G). A 2.1-kb BamHI-Xhol fragment containing PAK1 was cloned into BamHI-Sall-digested pHIPX4-B (Komori et al. 1997). The resulting plasmid was linearized with SphI and integrated into the genome of *H. polymorpha* NCYC495 *leu1.1*. Correct integration in the PAK1 locus as a single copy was confirmed by Southern-blot analysis (data not shown).

Biochemical methods. Crude extracts were prepared as described (Waterham et al. 1994). For the generation of protoplasts whole cells were pre-incubated in a solution containing 100 mM Tris pH 8.0,
50 mM EDTA, 140 mM β-mercaptoethanol and 1.2 M sorbitol for 15 min at 37°C. The cells were harvested by centrifugation, washed once in a 50 mM potassium phosphate buffer pH 7.2, containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mg ml⁻¹ of zymolyase 20 T for 15 min at 37°C. The cells were harvested by centrifugation, washed once in a 50 mM potassium phosphate buffer pH 7.2, containing 1.2 M sorbitol, and incubated in the same buffer containing 1 mg ml⁻¹ of zymolyase 20 T at 37°C for 15 min at 37°C. The resulting supernatant was subsequently centrifuged for 10 min at 4000 g. The resulting post-nuclear supernatant (PNS) was either centrifuged for 30 min at 30 000 g, in order to obtain an organellar pellet, or directly loaded onto a discontinuous sucrose density gradient (Douma et al. 1985). High-salt treatment of the organellar pellet was carried out as described by Komori et al. (1997). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard. Dihydroxyacetone kinase was assayed as described by Bystrykh et al. (1990). DHA (Bystrykh et al. 1990) and methanol concentrations (Verduyn et al. 1984) were assayed by established procedures. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Gels were used for Western blotting (Kyhse-Anderson 1984), and blots were decorated using the chromogenic or chemiluminescent Western blotting kit (Boehringer Mannheim, Germany) with specific antibodies against H. polymorpha proteins.

Generation of polyclonal Pak1p antibodies. A 1.8-kb NaeI-XbaI fragment of PKA1 (encoding amino acids 40–592) was cloned between the XmnI and XbaI sites of pMAL-C2 (New England Bio-labs), resulting in a fusion gene consisting of the E. coli malE gene and PKA1. Expression of the fusion gene in E. coli was induced by adding 1 mM of IPTG to exponentially growing cultures. After 2 h of incubation the cells were harvested and lysed using lysozyme. The fusion protein was recovered by centrifugation as inclusion bodies, which were used for immunization in rabbit (Harlow and Lane 1988).

Electron microscopy. Whole cells and spheroplasts were fixed and embedded in Epon 812 or Unicryl as described previously (Waterham et al. 1994). Ultrathin Unicryl-sections were labelled using polyclonal antibodies raised in rabbit and goat-anti-rabbit antibodies conjugated to gold according to the instructions of the manufacturer (Amersham, UK).

^31^P nuclear magnetic resonance (NMR) experiments. ^31^P NMR spectra were obtained at 121.49 MHz on a Bruker MSL 300 spectrometer according to the procedures detailed previously (Nicolaï et al. 1987).

Results

Characterization of the per6-210 mutant

The H. polymorpha per6-210 mutant has been identified within a collection of methanol-utilization-deficient (Mut-) strains detailed previously (Cregg et al. 1990; Titorenko et al. 1993). Cells of this strain normally grow on rich media (e.g. YPD, mineral media containing glucose, ethanol or glycerol). However, growth on methanol is fully impaired. Upon transfer of glucose-grown per6-210 cells to methanol-containing media, peroxisomes developed which displayed an aberrant morphology (data not shown). Electron microscopical analyses showed that, after a shift of cells from glucose to methanol-containing media and due to the import of induced peroxisomal proteins (e.g. alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase), the initial development of the organelles was virtually unaffected and occurred as described for WT cells. However, upon maturation of the organelles (during prolonged incubation) their surrounding membranes frequently became ruptured. This process was associated with the formation of several new small organelles, which were virtually intact and, since they grew, were able to import newly synthesized proteins. The remaining, damaged organelles were often subject to rapid autophagic degrada-
tion by the mechanisms detailed before for the turnover of redundant intact peroxisomes in wild-type *H. polymorpha* (Veenhuis et al. 1983; data not shown, compare Fig. 7). Alternatively, AO crystalloids, lacking a surrounding membrane, remained in the cytosol where they often fragmented (data not shown; compare Fig. 7).

Cloning of per6-210-complementing DNA

In order to clone the complementing DNA fragment, mutant per6-210 was transformed with a genomic *H. polymorpha* library. Among approximately 10,000 transformants obtained, four grew well on methanol-containing agar plates (Mut+ phenotype). These clones appeared to carry overlapping genomic inserts. One clone was selected which contained plasmid DNA with a genomic insert of 5.2 kb. The nucleotide sequence of the complementing portion of this fragment was determined and was deposited at GenBank (accession number AF061946). A BLASTN database search revealed that this fragment corresponds to a region in a large *H. polymorpha* DL1 genomic DNA fragment which was not further characterized, but was cloned by functional complementation of a dihydroxyacetone kinase (DAK)-deficient mutant (Tikhomirova et al. 1988; EMBL accession number X58862).

Analysis of the per6-210-complementing sequence revealed two ORFs (Fig. 1). ORF1 encodes a protein of 609 amino acids with a predicted mass of 65 kDa. The protein product was highly similar (34% identity) to the translation product of the *Citrobacter freundii* dhaK gene, encoding the enzyme dihydroxyacetone kinase (DAK; Daniel et al. 1995). In addition two *Saccharomyces cerevisiae* ORFs were found to display strong similarity to ORF1 (51% and 46% identity; SwissProt accession number P43550 and PIR accession number S48327). An alignment of these sequences is given in Fig. 2.

The second ORF encodes a protein of 592 amino acids with a calculated molecular weight of 66.1 kDa (Fig. 3). It showed sequence homology (32% identity) over the entire
length with the Neurospora crassa THI4 gene, which is involved in thiamine biosynthesis (Akiyama and Nakashima 1996). Also the putative protein products of three S. cerevisiae ORFs [YOL055 C (PIR S66740), YPR121 W (PIR S69014) and YPL258 C (PIR S65289)] are homologous over their entire length (31–37% identity). Their functions are, however, as yet unknown. The N-terminal half of the ORF2 protein product showed similarity to a bacterial protein involved in thiamine biosynthesis (33% identity), namely Salmonella typhimurium 4 amino-5-hydroxymethyl-2-methyl pyrimidine phosphate kinase (HMPP-kinase, Swiss Prot P55882; Petersen and Downs 1997). In addition, the C-terminal domain of the ORF2 protein was similar to Bacillus subtilis tenA, a putative regulatory component of protein secretion (20% identity; Swiss Prot P25052; Pang et al. 1991).

A hydrophobicity plot of the gene product of ORF2 predicts one possible membrane-spanning region (amino acids 262–292). At the N-terminus a degenerate form of the N-terminal peroxisomal targeting signal PTS2 (RV-X_5-QL) is present. This sequence is identical to the putative PTS2 of Trypanosoma brucei glycosomal aldolase (Clayton 1985). We designated ORF2 as PAK1 and its translation product as Pak1p.

ORF1 encodes the cytosolic enzyme dihydroxyacetone kinase

To test whether ORF1 indeed represented the H. polymorpha DAK gene, as suggested by sequence analysis, a strain was constructed in which ORF1 was disrupted (see Fig. 1). Cells of this strain grew normally on mineral media containing glucose, ethanol or glycerol, but were unable to grow on methanol. In addition, cells of this strain were unable to grow on dihydroxyacetone, a compound which requires DAK activity for its metabolism (de Koning et al. 1987). Control cultures of wild-type (WT) or a peroxisome-deficient mutant (Apex8; Waterham et al. 1994) grew well on DHA. The absence of DAK activity in this strain was confirmed biochemically by enzyme activity measurements which revealed that in crude extracts, prepared from cells of the ORF1 disruption strain grown on glucose or glycerol or incubated for 24 h in methanol-containing media, DAK activity was invariably absent. Based on these findings we conclude that ORF1 encodes the H. polymorpha DAK enzyme, and designated it as DAK.

The morphology of peroxisomes in cells of the strain in which ORF1 was disrupted (Δak) was similar to those of normal intact peroxisomes present in WT cells (data not shown). Immunocytochemistry revealed that peroxisomal matrix proteins were, as in WT controls, confined to the organellar matrix (data not shown). Peroxisome disassem-
bly or increased organellar turnover, typical for per6-210 cells, was never observed in \(D_{dak}\) cells. The subcellular location of DAK was determined by cell-fractionation experiments (Fig. 4). Sucrose-density centrifugation of a post-nuclear supernatant obtained from homogenates of methanol-grown WT \(H.\ polymorpha\) resulted in a clear separation of peroxisomes (marker protein AO, fraction 6), mitochondria (marker protein cytochrome c oxidase, fraction 16) and soluble proteins (fractions 23–25). As shown in Fig. 4, DAK activity was confined to the upper part of the gradient, which indicates that it is a cytosolic protein.

Characterization of a \(PAK1\) disruption mutant

In order to test whether the peroxisomal aberrations observed in per6-210 cells were related to a deficiency in the protein product of the second ORF \((PAK1)\), a \(PAK1\) disruption mutant was constructed (Fig. 1). Physiological studies revealed that the resulting strain \((\Delta pak1)\) grew normally on methanol and peroxisomes were formed in methanol-grown \(\Delta pak1\) cells. Occasionally, peroxisomal abnormalities like those observed in per6-210 cells were encountered, especially in cells from batch cultures in the late exponential and stationary growth phase. Under these conditions immunocytochemical experiments revealed that the peroxisomal matrix proteins were predominantly peroxisomal but were also present in the cytosol (Fig. 5A, AO; DHAS not shown). In WT control cells labelling was invariably confined to the peroxisomal matrix when the same antisera were used (data not shown; see Douma et al. 1985).

Pak1p is a low abundant, methanol-inducible protein

In order to obtain insight into the function of the \(PAK1\) gene product, the levels of Pak1p were determined in cells grown under various conditions. For this purpose, Pak1p antibodies were raised using a Pak1p-MBP fusion protein synthesized in \(E.\ coli\). With these antibodies, a single protein band of approximately 68 kDa was observed in Western blots prepared from crude extracts of methanol-grown \(H.\ polymorpha\) WT cells (Fig. 6A). The apparent molecular weight of the protein was in good agreement with the calculated mass of the protein (66.1 kDa). The intensity of the band was very low and only detectable using chemiluminescence techniques. In extracts prepared from \(\Delta pak1\) cells the 68 kDa-band was invariably absent. To increase the Pak1p protein level, a strain was constructed which contained an additional copy of \(PAK1\) under the control of the strong AO promoter. In Western blots of crude extracts prepared from methanol-induced cells of this strain, the intensity of the 68-kDa band had significantly increased. We concluded from these data that the 68-kDa band represented Pak1p and that the antiserum specifically recognized Pak1p, but no other \(H.\ polymorpha\) proteins.

The induction of Pak1p was subsequently analyzed in crude extracts of variously grown \(H.\ polymorpha\) WT cells by Western blotting using \(\alpha\)-Pak1p antibodies. The results, presented in Fig. 6B, indicate that synthesis of Pak1p is enhanced in methanol-grown cells compared to glucose-grown cells. In cells grown on glucose in the presence of thiamine, Pak1p was not detected, but a faint band became
visible when crude extracts were used prepared from cells grown on glucose under thiamine limitation. An increase in Pak1p levels due to thiamine limitation was more evident when cells grown on methanol under conditions of thiamine excess were compared with cells grown on methanol under thiamine-limitation conditions (Fig. 6B).

Subcellular location of Pak1p

Immunocytochemical experiments on ultrathin sections of WT H. polymorpha cells, using α-Pak1p antibodies, resulted in a specific labelling of the peroxisomal membrane. However, the labelling intensities were very low (generally 2–3 gold particles/organelle; Fig. 5B), in agreement with the very low levels of Pak1p in WT cells. The ultrastructural data, which suggested that Pak1p is associated with the peroxisomal membrane, could not be confirmed biochemically using cell-fractionation experiments. In sucrose gradients, prepared from a post-nuclear supernatant of methanol-grown WT cells, a very minor portion of Pak1p co-sedimented with the peroxisomes (fraction 6) while the bulk of the protein was found on top of the gradient (Fig. 4). Upon extraction of an enriched peroxisomal fraction (30 000 g pellet) by high salt, all sedimentable Pak1p became solubilized. This suggests that the organelar Pak1p is loosely associated with the outer surface of the organelar membrane (Fig. 6C). After over-production of Pak1p, α-Pak1p-specific labelling was predominantly localized at the peroxisomal membrane, but was also present in the cytosol (Fig. 5C). The latter results should however be interpreted with care, because over-production of Pak1p may alter its subcellular location. Taken together these data suggest that Pak1p may have a dual location and is present both at the peroxisomal membrane and in the cytosol.

Per6-210 is affected in both DAK and Pak1p

The phenotypes of the single Δdak and Apak1 mutants differed from that of the original per6-210 mutant. Analysis of the original mutant per6-210 revealed that this strain is unable to grow on DHA and lacked DAK activity, indicating that the DAK gene is inactivated. In addition, methanol-induced per6-210 cells contain strongly reduced Pak1p levels (Fig. 6D), which suggests that expression of PAK1 may be disturbed as well. Additional evidence that per6-210 is defective in both gene products came from
studies on a constructed double mutant in which major parts of both the DAK and PAK1 genes were deleted (Δdak Δpak1; Fig. 1). As expected, Δdak Δpak1 cells lacked DAK activity and were unable to grow on methanol or DHA. Also Pak1p was absent in these cells (Fig. 6D). Ultrastructural analysis revealed that the peroxisomal morphology in methanol-induced cells of the double mutant was similar to that of the original per6-210 mutant (Fig. 7).

A single mutation in per6-210 may affect both DAK activity and PAK1 expression.

The smallest DNA fragment still able to functionally complement the per6-210 mutation is a 3.2-kb PvuII fragment (nt 1357–4422) containing the 3' half of ORF1 (encoding the C-terminal 310 amino acids of DAK) and the complete ORF2 (see Fig. 1). Most likely, functional complementation was due to integration/recombination events. Sequence analysis of a cloned 1.1-kb fragment which included the 3' half of DAK and the 5' end of PAK1, revealed that in per6-210 a G-to-A transition had occurred in nucleotide 1658. This mutation resulted in the substitution of the conserved glycine 401 of DAK into glutamic acid (Fig. 2). This mutation is present only 670 bp upstream of the start codon of PAK1 and hence could affect the regulatory elements of the PAK1 promoter.

Taken together, these data support the view that possibly both genes may be inactivated in per6-210 due to a single mutation.
Physiological studies

To gain further insight in the role of DAK and Pak1p in methanol metabolism, physiological studies were carried out using glucose-limited chemostat cultures of Δpak1, Δdak or Δdak Δpak1 cells. In WT H. polymorpha the addition of methanol as a second carbon source to glucose-limited chemostat cultures results in an increase in yield due to the simultaneous utilization of glucose and methanol under these conditions (van der Klei et al. 1991). In experiments, in which 0.2% methanol was added to a glucose-limited chemostat culture of the Δdak Δpak1 double mutant the yield of the culture increased only slightly (0.4 D_{660} units). In similar experiments, carried out with the single mutant Δpak1, the increase in biomass was considerably higher (1.9 D_{660} units) and comparable to the values observed in cultures of WT cells (van der Klei et al. 1991). In case of the Δdak mutant an intermediate increase was observed (0.8 D_{660} units). Hence, the low increase observed in Δdak Δpak1 cultures most likely reflects the combined defects of both DAK and Pak1p. In vivo 31P NMR studies on methanol-induced cells demonstrated that the reduction in yields of Δdak Δpak1 cultures were not due to specific energetic disadvantages caused by damaged or non-functional peroxisomes, as have been observed in various pex strains (van der Klei et al. 1991). In both cultures normal ATP levels were observed and the inorganic phosphate peaks, indicating the acidic nature of the peroxisomal lumen (pH approximately 5.8–6.0), were normally found (data not shown, see Nicolay et al. 1987). These results therefore confirm the morphological data which indicated that in Δdak Δpak1 cells at least a substantial portion of the peroxisomal population is intact and physiologically active.

An alternative explanation could be that, due to the absence of DAK activity, the cells start to produce dihydroxyacetone (DHA). Indeed, after the addition of methanol up to 12 mM, DHA was detectable in the growth medium. Hence, part of the formaldehyde produced from methanol oxidation is assimilated by DHAS, resulting in the formation and subsequent secretion of DHA and, as such, can account for the drop in yield compared to WT controls.

The H. polymorpha DAK protein has been purified to homogeneity and characterized by Kato et al. (1988), who demonstrated that the enzyme was a homodimer composed of two identical subunits of 72 kDa, which is in fair agreement with the calculated molecular weight of the H. polymorpha DAK protein (65 kDa). In H. polymorpha, DAK is a cytosolic enzyme of the xylulose-5-phosphate (Xu5P) pathway, essential for the assimilation of formaldehyde generated from methanol. The first step in the assimilation pathway is the conversion of formaldehyde and Xu5P into dihydroxyacetone (DHA) and glyceraldehyde phosphate (GAP), mediated by peroxisomal dihydroxyacetone synthase (DHAS; Douma et al. 1985). Subsequently, DHA is phosphorylated in the cytosol by DAK, resulting in the formation of dihydroxyacetone phosphate (DHAP). Then, DHAP and GAP are converted into fructose-bis-phosphate (FBP) and subsequently, in a series of rearrangement reactions, into GAP, used for biosynthetic processes, and Xu5P, to replenish the Xu5P essential for the initial – peroxisomal – condensation reaction (van der Klei et al. 1991). Prevention of DHA phosphorylation in a Δdak strain inhibits operation of the Xu5P pathway (and thus growth on methanol as a sole carbon and energy source) and explains the Mut− phenotype and the observed secretion of DHA in methanol cultures of these strains.

As expected, in chemostat cultures of Δdak cells on glucose/methanol mixtures, methanol was fully used because AO was normally active. However, the addition of methanol as second substrate resulted in a significantly lower increase in biomass, as observed in WT controls (Δdak 0.8 and WT 2.0 D_{660} units, respectively). This can be explained by the fact that glucose-derived moieties have to replenish the DHAP, which cannot be formed by the normal Xu5P pathway in Δdak cells.

The effects of the Pak1p disruption on the efficiency of methanol metabolism and peroxisome integrity was particularly prominent in a Δdak Δpak1 double mutant during the growth of cells in a chemostat on glucose/methanol mixtures. Essentially, the phenotype of methanol-induced pertb-210 cells (Mut−, peroxisomal defects, no DAK activity) was akin to the constructed Δdak Δpak1 cells and thus most likely represents the combined effects of the disruption of either of the DAK or the Pak1 genes alone. This view is supported by the observed reduced Pak1p levels in pertb-210 cells which might be explained by a point mutation in the coding region of DAK, which supposedly overlaps with the promoter region of Pak1.

Discussion

In this paper we describe the H. polymorpha pertb-210 mutant, which is impaired in respect of growth on methanol (Mut−) and characterized by the presence of aberrant peroxisomes. The complementing DNA fragment contained two complete ORFs. ORF1 encodes the H. polymorpha enzyme dihydroxyacetone kinase (DAK), while ORF2 codes for a protein, designated Pak1p, which plays a role in peroxisome integrity, especially in the later stages of growth of cells on methanol. So far, only a single gene encoding DAK has previously been characterized, namely dhaK of the prokaryote Citrobacter freundii (Daniel et al. 1995), which shows 34% identity to the H. polymorpha protein.
grown cells. Also, \( \textit{PAK1} \) expression is partially repressed by thiamine, a phenomenon which has been reported for several, but not all, genes involved in thiamine biosynthesis (Begley 1996).

Although the above findings suggest a role of Pak1p in thiamine biosynthesis, this function is not immediately clear in the case of \( \textit{H. polymorpha} \) because this organism is not able to synthesize thiamine, but is dependent on the addition of this vitamin to the cultivation medium for growth. A clue to the function of Pak1p may be deduced from studies on thiamine biosynthesis in \( \textit{S. cerevisiae} \) and \( \textit{S. pombe} \). This view is based on the fact that the subcellular locations of the known yeast enzymes involved in thiamine biosynthesis are not yet known. However, TPP-dependent enzymes are present in various cell compartments (e.g. mitochondria, peroxisomes). This therefore implies that TPP (or precursor forms) are either transported across intracellular membranes or else synthesized in different subcellular compartments. Interestingly, in \( \textit{S. cerevisiae} \) three Pak1p homologues have been found which may be located at different sites in the cell. If so, it can be envisaged that, due to transport barriers, defects in the synthesis at one site can not be (fully) restored by thiamine added to the growth medium or synthesized in another organelle.

This may serve as an explanation as to why in \( \textit{H. polymorpha} \) a defect in a gene required for thiamine biosynthesis still shows a phenotype even when thiamine is supplemented in the growth medium. Decreased TPP levels in \( \textit{PAK1} \) disruption strains may explain the mislocation of a portion of the peroxisomal enzymes in the cytosol. The assumption that limitation of a co-factor of a peroxisomal enzyme may affect its subcellular location is not without precedent. Studies on a riboflavin (Rf)-auxotrophic mutant of \( \textit{H. polymorpha} \) showed that the limitation of Rf (and thus FAD, the AO co-factor) resulted in the mislocation of a portion of the AO protein and other peroxisomal matrix enzymes in the cytosol (Evers et al. 1994, 1996). A similar phenomenon may explain the observed cytosolic portion of peroxisomal enzymes in a Pak1p-deficient environment, namely an imbalance in the amount of newly synthesized DHAS protein and its co-factor TPP.

Taken these data together, we propose that Pak1p plays a role in thiamine biosynthesis and is possibly involved in the generation of adequate amounts of the co-factor TPP for the peroxisomal enzyme DHAS.

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