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Isolation of mutants of *Hansenula polymorpha* defective in the assembly of octameric alcohol oxidase

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Abstract

Alcohol oxidase (AO) is a peroxisomal enzyme that catalyses the first step in methanol metabolism in yeast. Monomeric, inactive AO protein is synthesised in the cytosol and subsequently imported into peroxisomes, where the enzymatically active, homo-octameric form is found. The mechanisms involved in AO octamer assembly are largely unclear. Here we describe the isolation of *Hansenula polymorpha* mutants specifically affected in AO assembly. These mutants are unable to grow on methanol and display reduced AO activities. Based on their phenotypes, three major classes of mutants were isolated. Three additional mutants were isolated that each displayed a unique phenotype. Complementation analysis revealed that the isolated AO assembly mutants belonged to 10 complementation groups. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Alcohol oxidase; Protein assembly; Peroxisome; *Hansenula polymorpha*

1. Introduction

Peroxisomes represent a class of organelles that are present in almost all eucaryotic cells. Peroxisomal matrix enzymes function in various metabolic processes [1], depending on the organism and/or their developmental state. In man their function is essential; peroxisome malfunctioning results in severe clinical symptoms and may lead to an early death [2]. In yeast cells, however, defects in peroxisome function do not affect viability but lead to severe physiological and energetic disadvantages under conditions that require the functioning of these organelles (e.g. growth of the organisms on methanol or oleic acid) [3]. These features have allowed to isolate various yeast mutants defective in peroxisome biogenesis and function (*pex* mutants) that have been a rich source for our current understanding of the principles of peroxisome assembly [4–7].

The yeast *Hansenula polymorpha* is able to use methanol as sole carbon and energy source. Growth on this compound is accompanied by a massive proliferation of peroxisomes, containing the key enzymes of methanol metabolism: alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT) [8]. AO is a homo-octameric flavoprotein of 600 kDa that contains a non-covalently bound flavin adenine dinucleotide (FAD) molecule as co-factor in each subunit. AO mediates the initial step in methanol catabolism [3].

AO enzyme activity is confined to the peroxisomal matrix. AO monomers are synthesised in the cytosol and post-translationally imported into the target organelle, where the assembly into active octamers is assumed to take place [3,9–11]. Routing of AO monomers from the cytosol to their target organelle is mediated by a cytosolic receptor, Pex5p, which interacts with a C-terminal peroxisomal targeting signal (PTS1) [12]. This receptor–cargo complex interacts with a putative docking complex at the peroxisomal membrane followed by translocation. Although some of the factors involved in this process have been identified, still little is known of the principles of the actual translocation process [2]. The same holds for

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the process of assembly and activation of complex matrix proteins, also including AO. The progress in this field is severely hampered by the fact that in vitro assembly of enzymatically active, octameric AO from its constituents so far has failed.

Several experiments indicated that the assembly of AO is not a spontaneous process, but requires the function of helper proteins. For instance, after synthesis in heterologous hosts like *Escherichia coli* and *Saccharomyces cerevisiae* [13] AO assembly was prevented, irrespective of the subcellular localisation of the protein. On the other hand, in peroxisome-deficient (*PEX*) mutants of *H. polymorpha* [4] various peroxisomal proteins, including AO, are normally assembled and active in the cytosol [14]. This indicates that putative peroxisomal helper proteins do not require the specific micro-environment of the peroxisomal matrix but can also function efficiently in the cytosol [14].

Studies on a riboflavin auxotrophic *H. polymorpha* mutant have indicated that limitation of the co-factor FAD interferes with both import and assembly of AO [11]. Possibly, FAD-lacking AO monomers accumulate in the matrix, where they remain bound to proteins involved in import/assembly due to the absence of the co-factor. As a result the protein import machinery may become stuck, resulting in a further block in matrix protein import. The apparent relation between co-factor binding and import of AO into peroxisomes is furthermore evident in mutants, in which the FAD-binding fold was modified by site-directed mutagenesis. Such mutant AO proteins show a reduced stability as well as import handicaps [15] and are not suitable for assembly studies.

Taken together, these data led us to conclude that (i) helper proteins exist that are involved in AO assembly and (ii) cytosolic accumulation of monomeric, FAD-lacking AO is indicative for specific AO assembly defects.

Therefore, we set out a molecular approach to identify specific factors involved in AO assembly. Here, we report the isolation of a collection of mutants of *H. polymorpha* that are specifically defective in AO assembly. These novel mutants are promising tools to unravel the molecular mechanisms involved in import/assembly of AO in detail.

2. Materials and methods

2.1. Organisms and growth conditions

The isogenic *H. polymorpha* strains NCYC 495 (*leu1-1*, *ade11-1*, *met6-1*, *ade11-1 met6-1* and *ura3-1*) [16] and NCYC 495 Δ AOX1-9 were used in this study. Wild-type (WT) and mutant strains were grown in batch cultures at 37°C on (a) selective medium, containing 0.67% (w/v) Yeast Nitrogen Base without amino acids (Difco), 1% (w/v) glucose (YND) or 0.5% (v/v) methanol (YNM); (b) YPD medium, containing 1% (w/v) yeast extract, 1% (w/v) peptone and 1% (w/v) glucose or mineral medium

[17] supplemented with 0.5% (w/v) glucose or 0.5% (v/v) methanol in the presence of 0.25% (w/v) ammonium sulfate as nitrogen source. For the induction of peroxisomes/peroxisomal enzymes, mutant strains were precultivated in YPD medium, diluted in methanol-containing mineral media and incubated for 24 h. When needed, amino acids were supplemented at the appropriate concentrations. For growth on agar plates, the media were supplemented with 1.5% agar.

2.2. Mutant isolation

Cells of *H. polymorpha* NCYC 495 (*leu1-1*) were mutagenised using *N*-methyl-*N'*-nitro-nitrosoguanidine (NTG), followed by nystatin enrichment and selection of mutants defective in methanol utilisation (Mut⁻ mutants) [4,18]. Mut⁻ strains were subsequently grown on YPD plates for 2 days at 37°C, replica-plated on plates containing mineral medium with 0.5% (v/v) methanol as the sole carbon source and incubated for another 24 h. These plates were screened using a colony plate assay [19] to visualise AO activity. Putative AO assembly-deficient mutants, which showed no or reduced AO activity relative to wild-type cells, were selected. These mutants were analysed for the induction of peroxisomes and enzymes involved in methanol metabolism, by phase contrast light microscopy and biochemical methods. Selected mutants were grown in the presence of riboflavin, the precursor of FAD, to exclude an affected FAD synthesis [11]. Mating, complementation analysis, sporulation of the hybrids and random spore analysis were performed by established procedures [16].

2.3. Biochemical methods

Crude extract preparation [12] and AO activity measurements [20] were performed as detailed before. AO octamer/monomer distribution was analysed by sucrose density centrifugation [10]. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis [21] and Western blotting were performed as described [22], using antiserum against various peroxisomal matrix and membrane proteins. Proteins on Western blots were detected using the chromogenic Western blotting kit (Roche, Almere, The Netherlands).

2.4. Electron microscopy

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described [23]. Immunolabelling was performed on ultrathin sections of Uni-cryl-embedded cells, using specific polyclonal antibodies against various *H. polymorpha* proteins and gold-conjugated goat anti-rabbit (GAR) antibodies [23]. Cytochem-

ical staining experiments for the detection and localisation of AO activity were performed by the $CeCl_3$ -based method [24].

3. Results

3.1. Strategy for the isolation of alcohol oxidase assembly-deficient mutants

Since AO is the first enzyme in methanol metabolism, we anticipated that AO assembly-deficient (Ass^-) mutants of *H. polymorpha* are unable to utilise methanol as sole source of carbon and energy (Mut^- phenotype). Basically two Ass^- related defects were expected: (1) mutants defective in specific cytosolic proteins (e.g. molecular chaperones) involved in AO import and/or assembly. In these mutants AO monomers are likely to accumulate in the cytosol. (2) Mutants defective in a peroxisome-bound process related to AO import/assembly, which most likely also show – indirectly – a block in AO import. For both types of mutants, cytosolic accumulation of AO monomers may occur eventually in conjunction with low amounts of peroxisomal, active AO in case of less severe mutations.

These assumptions imply that we had to select Mut^- mutants that contained little or no AO activity, in conjunction with distinct amounts of monomeric AO protein.

3.2. Isolation of Mut^- mutants affected in AO activity

Following NTG mutagenesis, 2000 Mut^- mutant strains were isolated. To identify putative Ass^- mutants within this Mut^- collection we performed a colony plate assay for the detection of AO activity. In this way 210 mutants that either completely lacked (Aox^-) or displayed strongly reduced AO activities ($Aox^{+/-}$), relative to WT controls, were identified. These mutants were backcrossed using isogenic *H. polymorpha* strains with different auxotrophic markers as well as a strain in which the *AOX* gene was disrupted to weed out mutants affected in the structural *AOX* gene. Numerous Aox^- strains appeared to be affected in the structural *AOX* gene, identified by the diploid Aox^- phenotype in the cross-combinations tested. In addition, several mutants were unable to mate or affected in FAD synthesis and were therefore also excluded. Ultimately, 36 stable Aox^- and 34 $Aox^{+/-}$ mutant strains

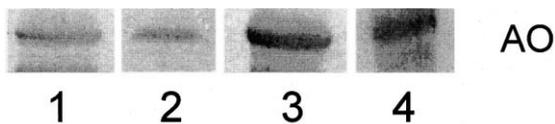


Fig. 1. AO protein levels in representative strains of class 1 (lane 1), class 2 (lane 2) and class 3 (lane 3) mutants and in WT *H. polymorpha* (lane 4). Western blots were prepared from cell-free extracts prepared from methanol-induced cells. The blots were decorated with antibodies against *H. polymorpha* AO. Equal amounts of protein were loaded per lane.

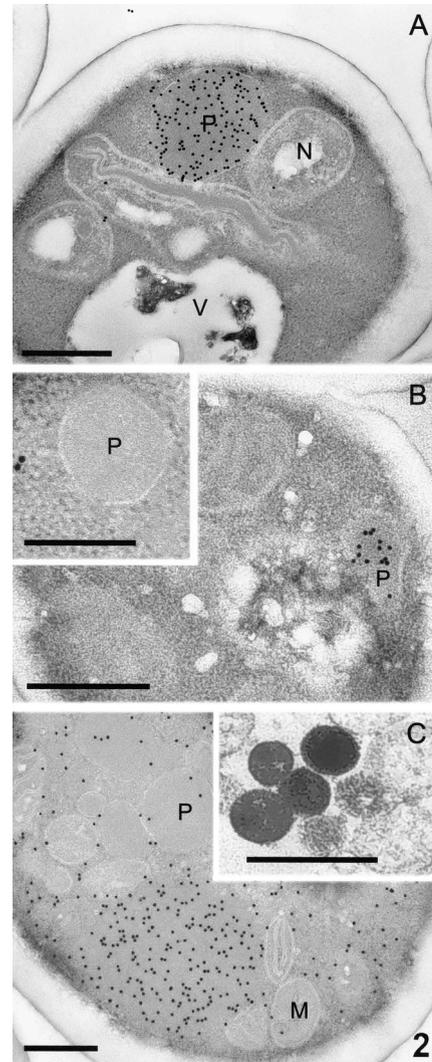


Fig. 2. Ultrastructure and protein localisations in representative strains of class 1, 2 and 3 Ass^- mutants. Figure A shows the presence of one large peroxisome in a methanol-induced cell of a representative of class 1 mutants. Immunolabelling using anti-AO antibodies revealed that all AO protein was properly transported into this single organelle. In Figure B a thin section of a representative strain of the class 2 mutants is shown. Upon labelling with anti-CAT antibodies, labelling is found throughout the peroxisomal matrix. These peroxisomes lack AO as evident from immunolabelling using anti-AO antibodies (inset). Figure C gives an example of a class 3 mutant. Using anti-AO antibodies labelling was predominantly found in the cytosol, whereas relatively low amounts were found in peroxisomes. The inset shows an AO activity staining of a cell of the same mutant, indicating that AO activity was confined to the small peroxisomes. The electron micrographs are of aldehyde-fixed cells. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 0.5 μm .

were selected and grown in shake flask cultures for further analysis. To this end, the strains were precultured on glucose, transferred to fresh methanol-containing media and harvested after 24 h for AO activity measurements, Western blotting and electron microscopy.

The results indicated that the AO activity plate assay was not fully reliable. We observed several mutants that contained normal WT AO activities after cultivation in

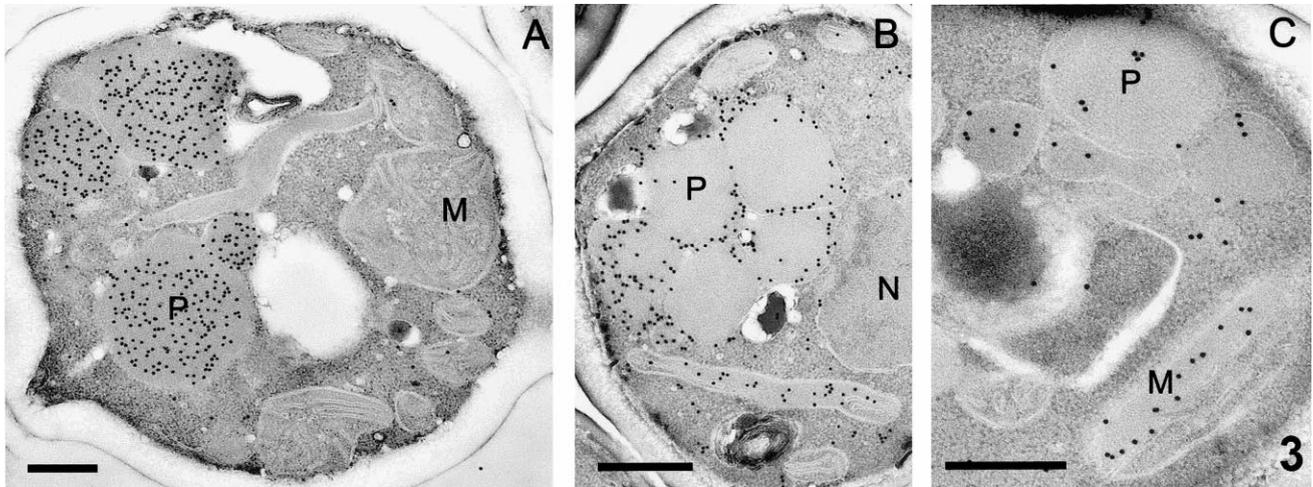


Fig. 3. Immunocytochemical localisation of AO and CAT in *Ass⁻* mutants 11 and 25. Panel A shows the localisation of AO in methanol-induced cells of mutant No. 11. AO-specific label is exclusively present in peroxisomes. In the same mutant CAT-specific label (B) is found at peroxisomes, mitochondria and in the cytosol. Methanol-induced cells of mutant 25 showed a similar distribution of CAT (C). Aldehyde-fixed cells. M, mitochondrion; N, nucleus; P, peroxisome. Bar = 0.5 µm.

batch cultures. Also, these cells contained normal peroxisomes. These and other strains that showed growth defects not related to methanol utilisation were excluded from further analysis. Also several *pex* mutants were identified, which were excluded from these studies as well. The majority of the remaining mutants could be subdivided into three classes with respect to their AO protein contents (determined by Western blotting), AO enzyme activities and morphological characteristics.

Class 1 mutants displayed low AO protein levels (Fig. 1, lane 1), low AO-specific activities (approximately 10–20% of WT cells) and reduced numbers of peroxisomes. Immunocytochemical analysis showed that AO (and DHAS) was normally imported into peroxisomes (Fig. 2A). Anti-CAT-specific labelling was observed in the cytosol and on mitochondria.

Class 2 mutants also showed low AO protein levels (Fig. 1, lane 2), but without any detectable activity. The one or

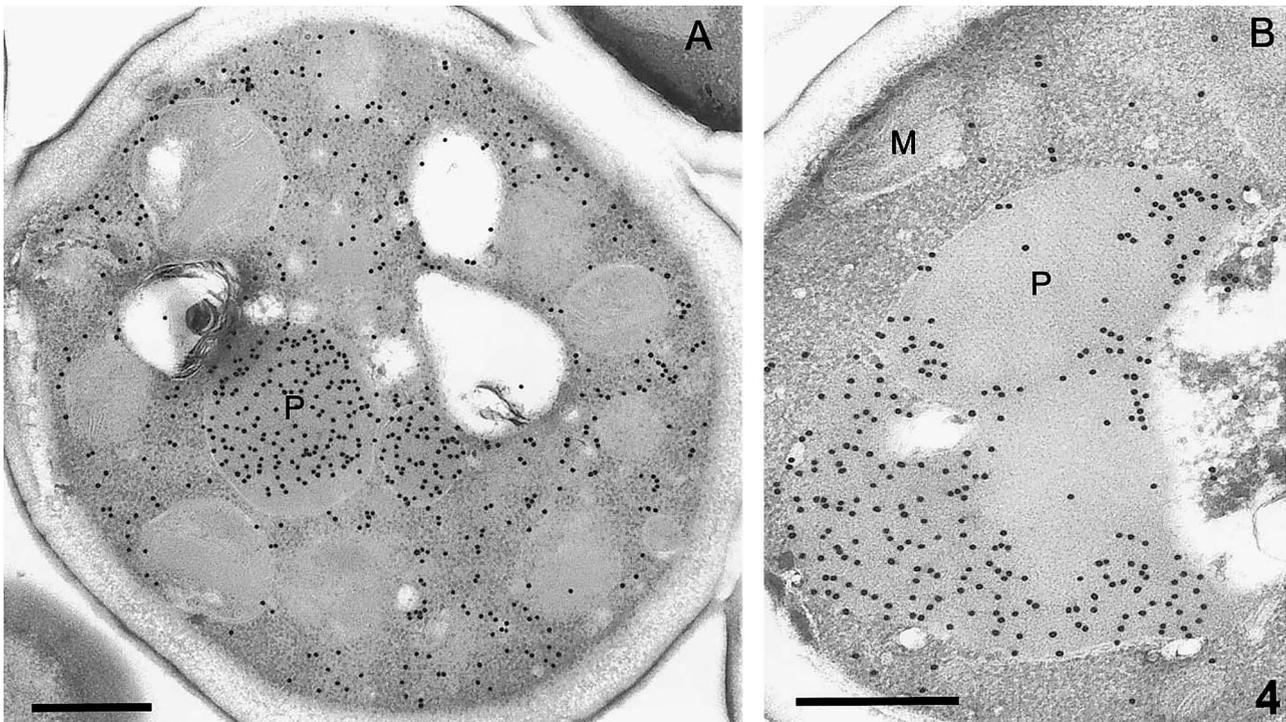


Fig. 4. Immunocytochemical localisation of AO and CAT in mutant 15. In methanol-induced cells of mutant 15 anti-AO-specific labelling was found in peroxisomes and cytosol (A). Using anti-CAT antibodies (B) labelling was predominantly found in peroxisomes. However, in these organelles CAT was randomly distributed throughout the organellar matrix. Aldehyde-fixed cells. M, mitochondrion; P, peroxisome. Bar = 0.5 µm.

two peroxisomes that were observed in these cells contained CAT (Fig. 2B), but lacked AO (inset Fig. 2B) and DHAS protein (not shown), which were solely detectable in the cytosol.

Class 3 mutants showed normal AO protein levels (Fig. 1, lane 3) but highly reduced specific AO activities, approximately 5–10% of that of WT controls. The mutant cells contained normal numbers of peroxisomes, though of smaller size than in WT cells. Immunocytochemically, α -AO-specific label was found in the cytosol, with only very little label on the peroxisomal profiles (Fig. 2C). Cytochemically, AO activity could be detected in the peroxisomal matrix but not in the cytosol (Fig. 2C inset). In these strains CAT and DHAS protein were confined to peroxisomes (data not shown).

Sucrose density centrifugation of a representative member of the three main classes of mutants confirmed the observed cellular distribution of AO and its activity. Methanol-induced cells of class 1 consisted mainly of (low amounts) octameric AO while the representative members of classes 2 and 3 showed mainly monomeric AO (not shown, van Dijk et al., in preparation).

Complementation analyses revealed that the members of the class 3 mutants all fell in one complementation group. In addition one member showed dominance over other members (2) of this class, most likely due to interallelic complementation. Six additional complementation groups were identified within class 1 and 2 mutants. Interestingly, members of classes 1 and 2 may fall in one and the same complementation group, indicating that the ultimate morphological and biochemical defect may be allele specific.

Three additional mutants were identified that showed marked deviations in AO and CAT protein location, namely mutants Nos. 11, 15, and 25. In methanol-induced cells of mutant No. 11 AO protein was detected in peroxisomes (Fig. 3A); CAT, however, was observed in peroxisomes, the cytosol but also in mitochondria where it was present in elongated aggregates (Fig. 3B). In mutant No. 15 AO protein was in both the cytosol and peroxisomes (Fig. 4A), whereas CAT was normally present in peroxisomes but showed an aberrant intra-organellar distribution in that the α -CAT-dependent labelling was present over a broad zone of the organellar matrix (Fig. 4B). This is clearly indicative for a defect in AO assembly since in WT peroxisomes CAT is invariably located at the edge of the organelle surrounding the AO crystalloid. Similar results were obtained for CAT localisation in cells of mutant No. 25 (Fig. 3C). Moreover, also AO protein was observed in the matrix of mitochondria of these cells.

Complementation analysis revealed that mutants Nos. 11, 15 and 25 fell in separate complementation groups, which were different from the seven complementation groups of the mutants in classes 1–3. In total therefore 10 different complementation groups have been identified in this study.

4. Discussion

We have isolated and characterised a collection of mutants (Ass^- mutants) of the yeast *H. polymorpha* that are affected in the assembly of the peroxisomal matrix protein AO. These mutants were isolated from a collection of strains that were impaired to utilise methanol as sole source of carbon and energy (Mut^- phenotype). Unexpectedly, the initial classification as AO activity-deficient by the colony plate assay was not completely reliable. In some of the mutants significant AO activities were detected in cells, incubated in liquid, methanol-containing media. Apparently, these cells displayed some resistance towards digitonin, used as the permeating agent in the plate assay.

It is interesting to note that the complementation analysis revealed complementation groups that contained members of different subclasses as they were identified on the basis of morphological and biochemical properties. This indicates that the actual Ass^- phenotype may be allele specific. This is in line with previous data on *H. polymorpha pex* strains which showed that different mutations in the same gene may lead to different mutant phenotypes [25].

The observation that some Ass^- mutants contained only a single peroxisome suggests that the proliferation of peroxisomes is hampered in these strains. Most likely this is not due to decreased levels of import-competent AO protein since also in constitutive *pex* mutants a correlation between peroxisome numbers and AO protein levels was never observed. Instead, reduced AO levels only affected the size of the individual organelles, but not the number. All observations made so far support the view that peroxisome proliferation (and thus, their numbers per cell) is not dependent on the rate of uptake of matrix proteins but is determined by physiological parameters [26,27]. Since these organelles were indistinguishable from peroxisomes in glucose-grown cells, a possible explanation for their appearance is that in these mutants the signal transduction pathway is affected that controls the cellular switch from non-methylotrophic to methylotrophic growth conditions.

Another unexpected finding was the localisation of peroxisomal matrix enzymes in the mitochondrial matrix. This has been shown before for alanine/glyoxylate aminotransferase (AGT), in which different point mutants have been described that resulted in altered AGT folding and/or the introduction of a mitochondrial-type targeting signal and – as a consequence – mislocalisation of this peroxisomal protein to mitochondria [28,29]. However, in case of AO the mislocalisation to mitochondria cannot be due to a defect in the AO gene since such mutants have been weeded out from the initial collection. Also, sequence analysis has never revealed any putative mitochondrial targeting signal in CAT or AO. In *H. polymorpha* thus far only constituents of the peroxisomal membrane pro-

teins, e.g. Pex14p, were detected in mitochondria of mutant (*pex*) cells. Theoretically, therefore also other components of the peroxisomal import machinery could have accumulated at the mitochondria, thus explaining the partial import of the matrix proteins. Another possible clue may be related to the recent findings that the PTS1 may not be the only determinant that prescribes peroxisomal sorting of AO and CAT [30,31]. It could be speculated that other cytosolic factors are required to mediate import, in the absence of which these proteins are maintained in a specific – probably unfolded – conformation in the cytosol that renders them accessible, by an as yet unknown mechanism, to the mitochondrial import machinery. If true, these mutants could be very helpful to study the early events of AO – and eventually CAT – assembly.

The severe import defect of AO – but not of CAT and DHAS – observed in class 3 mutants suggests that in these cells the PTS1 protein import pathway was normally functional. This led us to speculate that the intrinsic failure in AO assembly itself may have caused the import defect of the protein. A similar phenomenon has been observed for AO in auxotrophic riboflavin mutants during riboflavin limitation [11]. That this in fact may be true is evident from the data obtained from the initial cloning of genes of selected strains of different complementation groups. Three *ASS* genes have now been cloned, one of which has been characterised in detail (van Dijk et al., in preparation). The protein product of this gene, pyruvate carboxylase, appeared to possess a dual function. As an enzyme it showed the expected anapleurotic function, while the protein – and not the enzyme activity – was essential for AO assembly in that it may facilitate FAD binding to AO monomers prior to import.

This is the first example of a specific protein that is uniquely involved in AO sorting and assembly and as such a good example of the potential of our mutant approach.

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