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Cytosolic aspartate aminotransferase encoded by the *AAT2* gene is targeted to the peroxisomes in oleate-grown *Saccharomyces cerevisiae*

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Fatty acid β -oxidation in peroxisomes requires the continued uptake of fatty acids or their derivatives into peroxisomes and export of β -oxidation products plus oxidation of NADH to NAD. In an earlier study we provided evidence for the existence of an NAD(H) redox shuttle in which peroxisomal malate dehydrogenase plays a pivotal role. In analogy to the NAD(H)-redox-shuttle systems in mitochondria we have investigated whether a malate/aspartate shuttle is operative in peroxisomes. The results described in this paper show that peroxisomes of oleate-grown *Saccharomyces cerevisiae* contain aspartate aminotransferase (AAT) activity. Whereas virtually all cellular AAT activity was peroxisomal in oleate-grown cells, we found that in glucose-grown cells most of the AAT activity resided in the cytosol. We demonstrate that the gene *AAT2* codes for the cytosolic and peroxisomal AAT activities. Disruption of the *AAT2* gene did not affect growth on oleate. Furthermore β -oxidation of palmitate was normal. These results indicate that *AAT2* is not essential for the peroxisomal NAD(H) redox shuttle.

Keywords: peroxisome; aspartate aminotransferase; NAD(H) redox shuttle; subcellular localization; *Saccharomyces cerevisiae*.

In the last few years much has been learned about the functional properties of peroxisomes and it is clear that they are involved in a variety of metabolic pathways. Oxidation of fatty acids is a characteristic function of peroxisomes in all species. Since peroxisomes are the exclusive site for fatty acid β -oxidation in *Saccharomyces cerevisiae*, transport of fatty acids or their fatty acyl-CoA esters into peroxisomes is required. For long-chain fatty acids this is mediated by the peroxisomal ATP-binding-cassette transporters Pxa1/Pxa2 (Hettema et al., 1996; Shani et al., 1995, 1996a, b; Swartzman et al., 1996). Previous studies have shown that the peroxisomal membrane is impermeable for the end products of fatty acid β -oxidation, such as acetyl-CoA (Elgersma et al., 1995). Accordingly, acetyl-CoA can only leave the peroxisome in the form of glyoxylate-cycle intermediates after condensation with oxaloacetate, or as carnitine ester, a reaction mediated by carnitine acetyltransferase (Elgersma et al., 1995). Furthermore, fatty acid β -oxidation in peroxisomes requires continuous oxidation of NADH, generated in the third step of β -oxidation. Recent studies have shown that peroxisomal malate dehydrogenase (MDH) is involved in the intraperoxisomal oxidation of NADH (Van Roermund et al., 1995), probably

as part of a redox shuttle. The other components of such a presumed redox shuttle remain to be identified.

Studies in the 1960s established that reoxidation of NADH generated in the cytosol is brought about by various redox shuttles, including the malate/aspartate shuttle, which involves the participation of cytosolic and mitochondrial forms of MDH and aspartate aminotransferase (AAT). A possibility would be that peroxisomal MDH is part of a similar malate/aspartate shuttle in peroxisomes (Elgersma and Tabak, 1996). Only two AAT have been reported in *S. cerevisiae*, one in mitochondria and one in the cytosol (Morin et al., 1992; Cronin et al., 1991). Inspection of the published amino acid sequence of the presumed cytosolic AAT reveals the tripeptide Ala-Lys-Leu at its C terminus, which is a known peroxisomal targeting signal (PTS) (McNew and Goodman, 1994; Elgersma et al., 1995). This raised the question of whether the presumed cytosolic *AAT2* may be localized in peroxisomes, possibly taking part in a malate/aspartate redox shuttle. The results of these studies are reported in this paper.

MATERIALS AND METHODS

Strains and culture conditions. The yeast strains used in this study were *S. cerevisiae* BJ1991 (*MATa, leu2, trp1, ura3-251, prb1-1122, pep4-3*) (as wild-type strain for gene disruptions and gradients) and *pas21* (*PAS21::LEU2, MATa, leu2, trp1, ura3-251, prb1-1122, pep4-3*) and *pex13* (*PEX13::LEU2, MATa, leu2, trp1, ura3-251, prb1-1122, pep4-3*) (as control strains without functional peroxisomes). The *Escherichia coli* strain used for cloning procedures was DH5 α F' (Raleigh et al., 1988).

The selection of yeast mutants was carried out on minimal glucose medium (medium A) containing 0.67% yeast nitrogen base without amino acids (Difco), 2% or 0.3% glucose, and amino acids if needed (20 μ g/ml L-tryptophan, 20 μ g/ml uracil,

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Abbreviations. AAT, aspartate aminotransferase; CTA1, peroxisomal catalase; HAD, 3-hydroxyacyl-CoA dehydrogenase; MDH, malate dehydrogenase; PGK, phosphoglycerate kinase; SDH, succinate dehydrogenase; PTS, peroxisomal targeting sequence; PGI, phosphoglucose isomerase.

Enzymes. Aspartate transaminase (EC 2.6.1.1); catalase (EC 1.11.1.6); 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); malate dehydrogenase (EC 1.1.1.37); glucose-6-phosphate isomerase (EC 5.3.1.9); succinate dehydrogenase (EC 1.3.99.1).

30 µg/ml L-leucine) or on rich galactose medium (medium B) containing 2% galactose, 1% yeast extract and 2% peptone. Rich yeast mediums used contained 0.5% potassium phosphate pH 6.0, 0.3% yeast extract, 0.5% peptone, and either 3% glucose, 3% potassium acetate, 3% glycerol, or 0.12% oleic acid/0.2% Tween-40. Oleic acid plates contained 0.1% oleic acid, 0.4% Tween-40, 0.1% yeast extract (Difco), amino acids as needed (20 µg/ml L-tryptophan, 20 µg/ml uracil; 30 µg/ml L-leucine) and 2% agar. For the *aat2*-disruption mutant, 100 µg/ml aspartate was added to the minimal medium. In this study fresh yeast cells were grown for at least 24 h on 0.3% minimal glucose and were shifted in the exponential growth phase to rich glucose, acetate, glycerol or oleate medium. The cultures were inoculated at such an A_{600} that after incubation for 15 h an A_{600} of 0.7–1.0 was reached. For growing *aat2*-disruption mutants, cells were picked from a fresh glycerol plate, since we observed that the *aat2*-disruption mutant has a higher frequency of forming petite mutants than the parental strain.

Cloning procedures. The *AAT2* gene was obtained by PCR on genomic DNA, with the following degenerate primers: 5'-GA(T/C)GA(T/C)AA(T/C)GG(G/A/T/C)AA(A/G)CC(G/A/T/C)TGGGT-3' (based on the amino acid sequence DDNGKPWW) and 5'-ACCAT(A/G)TC(T/C)TT(A/G)TGCCA(T/C)TG(T/C)-TC-3' (based on the amino acid sequence VMDKHWQE) (primer set 1). The annealing temperature used was 55°C. The obtained 833-bp fragment was cloned in the *HincII* site of pUC19 (resulting in pEL74) and sequenced. To construct a disruption plasmid, the *EcoRI/SphI* fragment of pEL74 was subcloned into the *EcoRI/SphI* site of pBR322. This resulted in pEL84 with a unique *HindIII* site in the *AAT2* gene fragment. This site was used to insert the *LEU2* gene (pEL86) or the Tn5 bleomycin-resistance gene (*BLE*), under the control of the *GAL4* promoter (pEL89).

Disruption of the *AAT2* gene. The disruption plasmid pEL89 (*AAT2::GAL-BLE*) was used as template for PCR with the primers 5'-GGTATTACCGGTCTGCCAAGTT-3' (based on the amino acid sequence GITGLPSL) and 5'-CGTTAATTCTGGCGTTTCCAAC-3' (based on the amino acid sequence TLEPTELL) (primer set 2). The obtained product was used to transform the wild-type strain BJ1991. Transformed cells were grown for 5 h on medium B, and incubated with 20–100 µg/ml phleomycin (CAYLA) for 2 h. Cells were plated on YPD plates. Genomic DNA was isolated from the obtained phleomycin-resistant colonies, and analyzed for *AAT2* gene disruptions by PCR and Southern blotting.

Construction of plasmids. The PGK (phosphoglycerate kinase) promoter expressing plasmid was made as follows. A *NarI-SacI* fragment containing the PGK promoter was cloned in the *NarI/SacI* site of pEL30 (Elgersma et al., 1993). The MDH-expressing plasmid pNV8 was made by cloning of the *SacI-HindIII* fragment (pEL143) containing the haemagglutinin-tagged MDH3 coding region (Elgersma et al., 1996a) in the *SacI-HindIII* site of the PGK promoter expression plasmid.

The long construct (pAAT2long) was obtained by PCR on genomic DNA of *S. cerevisiae*, with the primers 5'-TTTGATCCATGAACAAAAGAACACAGGAATACAAGAAC-3' and 5'-TTTCTGAGTTACAATTTAGCTTCAGTAGCATAGAGCGC-3'. The short construct (pAAT2short) was obtained by PCR on genomic DNA of *S. cerevisiae*, with the primers 5'-TTTGATCCATGTCTGCCACTCTGTTCAATAACATCG-3' and 5'-TTTCTGCAGTTACAATTTAGCTTCAGTAGCATAGAGCGC-3'. After digestion of the created *BamHI* and *PstI* sites, the fragments were cloned into the *BamHI/PstI* sites of YCPGK (with an N-terminal haemagglutinin tag). The long and short constructs were transformed to the *aat2*-disruption mutant. The transformants were rescued on minimal glucose me-

dium without aspartate. The average value of three separate clones was used. For electron microscopy one complementing clone was used.

Sequencing. The 833-bp fragment was sequenced with the Promega DNA sequence kit. Primer set 2 was chosen to construct a 729-bp fragment, which was used as a probe to isolate the *AAT* gene (*AAT2*) from a yeast genomic library in the multicopy vector YEp13 (Nasmyth and Tatchell, 1980). Analysis of the purified plasmids from positive colonies was carried out by PCR and restriction enzyme digests. Further sequencing of *AAT2* was carried out with the T7 DNA sequencing kit (Promega). Both strands were sequenced.

Northern blot analysis. mRNA of wild-type yeast grown on rich glucose, oleate, glycerol or acetate was isolated as described by Maccacchini (1979). 10 µg of RNA (measured at 260 nm) of each growth condition were subjected to gel electrophoresis and after overnight blotting on Hybond-N (Amersham) the blot was hybridized with a *AAT2* probe (PCR product of primer set 2), a peroxisomal catalase (*CTA1*) probe [*SalI-HindIII* fragment from p525 (kindly provided by A. Hartig)] or a *PEX13* probe (*KpnI* fragment from p20.11), labeled with [³²P]dATP via random priming.

Other DNA techniques. Standard DNA techniques were carried out as described (Sambrook et al., 1989). DNA isolation from agarose gels was carried out with the Prep A gene DNA purification kit from Biorad.

Subcellular fractionation and Nycodenz gradients. Subcellular fractionations were performed as described by Van der Leij et al. (1992). The protoplast-free nuclei-free organellar pellet was applied to a 15–35% continuous Nycodenz gradient (12 ml), with a cushion of 0.5 ml 50% Nycodenz, dissolved in 5 mM Mes pH 6.0, 1 mM EDTA, 1 mM KCl and 8.5% (mass/vol.) sucrose. The sealed tubes (quick-seal; Beckman) were centrifuged for 2.5 h in a vertical rotor (MSE 8*35) at 19000 rpm (29000 g). Fractions were collected.

Western blotting. Proteins were separated on 12% SDS/polyacrylamide gels and transferred to nitrocellulose paper in 25 mM Tris, 192 mM glycine and 20% methanol. The blots were blocked by incubation in Tris/NaCl/Tween-20 (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20) supplied with 2% non-fat dry milk. The same buffer was also used for incubation with the primary antibodies and for IgG-coupled alkaline phosphatase. The blots were stained in buffer A (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) with X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride as per manufacturer's instructions (Boehringer Mannheim).

Electron microscopy. Oleate-grown and glucose-grown cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Ultra-thin sections were prepared as described by Gould et al. (1990).

Antibodies. The NH-antibody was kindly provided by P. van der Sluijs, University of Utrecht, Utrecht, The Netherlands. The thiolase antibody was described by Elgersma et al. (1996b)

Digitonin titration. 1.5-ml Eppendorf tubes were filled with 360 µl 1.2 M sorbitol, 50 mM potassium phosphate pH 7.0, 1 mM EDTA, 1 mM KCl and increasing amounts of digitonin, and were heated to 30°C. 40 µl protoplast (0.35 mg/ml protein), isolated as described by Van der Leij et al. (1992), were added to the medium and after 10 min the incubation was stopped by adding 400 µl ice-cold (4°C) medium without digitonin. The aliquots were centrifuged for 5 min in an Eppendorf centrifuge at 13000 rpm. The supernatants were collected and the pellets were suspended in the medium described above. Several enzymes were measured in the pellet and supernatant fractions.

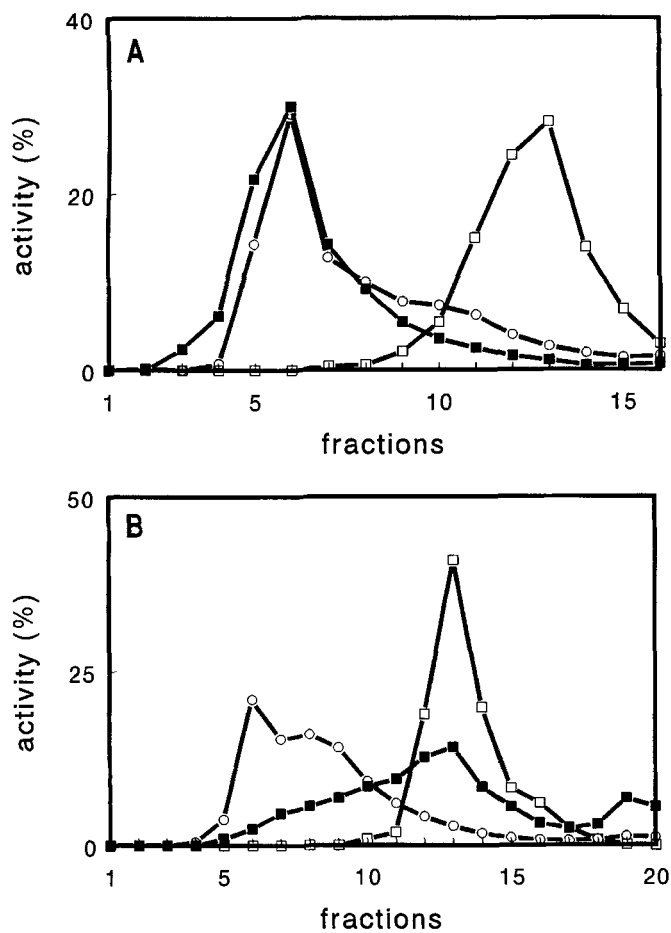


Fig. 1. Subcellular localisation of AAT in oleate-grown *S. cerevisiae*. An organellar pellet was obtained by subcellular fractionation of oleate-grown cells and used for density-gradient centrifugation on Nycodenz. This pellet, containing more than 85 % of total cellular activity of HAD, SDH and AAT, was taken up in medium (see Materials and Methods) and layered on top of the gradient. The absolute AAT activity (■) layered on the gradient of the *aat2*-disruption mutant (B) was 200-fold lower compared with the absolute AAT activity (■) layered on the wild-type gradient (A) (see Table 1 for absolute activities in cell free extracts). Fraction 1 represents the bottom of the gradient. SDH (□) and HAD (○) were measured as mitochondrial and peroxisomal marker enzymes, respectively.

Cell-free extracts, enzyme and protein assays. Cell-free extracts were obtained by breaking cells with glass beads in a medium containing 100 mM potassium phosphate pH 7.0, 1 mM phenylmethylsulfonyl fluoride and 0.025 % Triton X-100.

Fatty acid β -oxidation in intact cells was measured as described by Van Roermund et al. (1995), 3-hydroxacyl-CoA dehydrogenase (HAD) was measured as described by Wanders et al. (1990), catalase was measured as described by Bergmeyer et al. (1983b), succinate dehydrogenase (SDH) was measured according to Munujos et al. (1993), phosphoglucose isomerase (PGI) was measured essentially as described by Bergmeyer (1983c), AAT was measured as described by Bergmeyer (1983a), protein was either measured with the method of Smith et al. (1985), or with the Lancer Microprotein Rapid Stat Diagnostic kit, based on a method described by Bradford (1976).

RESULTS

Identification of AAT in peroxisomes. To investigate the involvement of AAT in a peroxisomal redox shuttle, we studied

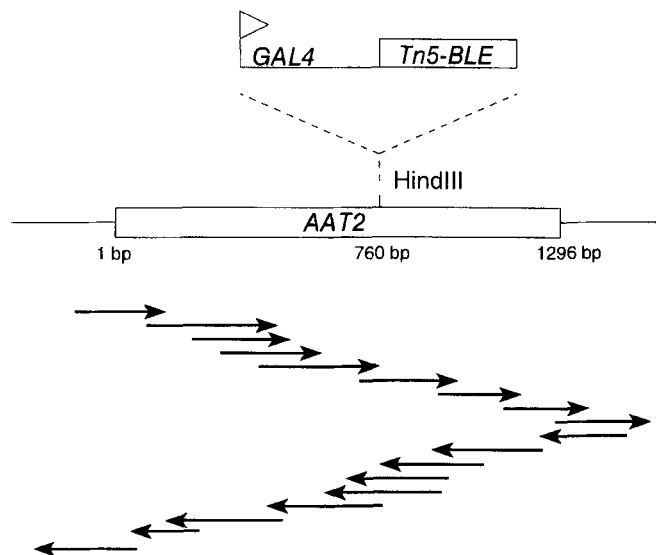


Fig. 2. Sequence strategy and disruption of the AAT2 gene.

whether or not peroxisomes from *S. cerevisiae* contain AAT activity. To this end we prepared an organellar fraction from oleate-grown *S. cerevisiae* cells, which was found to contain more than 85 % of total cellular activity of HAD, SDH and AAT. This fraction was subjected to density-gradient centrifugation.

Fig. 1A shows a good resolution between mitochondria and peroxisomes as reflected in the different peaks of activity for SDH and HAD as marker enzymes for mitochondria and peroxisomes, respectively. Furthermore we observed that AAT shows a similar distribution as the peroxisomal marker HAD, implying that peroxisomes contain AAT activity (Fig. 1A).

Cloning and sequencing of the AAT2 gene. Since the amino acid sequence of the AAT2 gene revealed a C-terminal putative PTS (AKL), this protein was a good candidate for the observed peroxisomal AAT activity. Therefore, based on the amino acid sequence of AAT2 (Cronin et al., 1991), we designed two degenerate oligonucleotides to clone the gene. The 833-bp fragment obtained upon PCR on isolated genomic DNA of wild-type cells was partly sequenced to identify the PCR product, and used to screen a yeast genomic library (Nasmyth and Tatchell, 1980). The clone obtained from the library was used for further sequencing (Fig. 2). Cronin et al. (1991) reported the purification of cytosolic AAT (AAT2). The amino acid sequence was determined, showing 414 amino acids, of which the amino acids on positions 232 and 233 were unknown. Our own sequence results, however, suggested a total length of the protein of 432 or 417 amino acids, depending on which methionine is used as a starting codon, since 14 amino acids in front of the first amino acid suggested by Cronin and colleagues another methionine is found (Fig. 3).

Comparing the amino acid sequence reported here with the published one (Cronin et al., 1991), revealed identity except that we found five amino acids at the location where Cronin and colleagues described two unknown amino acids on positions 232 and 233 (Fig. 3). These results suggest that, at least under glucose conditions, as used by Cronin et al. (1991), the second ATG is the codon at which translation starts.

Comparing our sequence with the AAT2 sequence of the genome-sequencing project from *S. cerevisiae*, we found four base-pair substitutions, resulting in two different amino acids (bold and underlined in Fig. 3). These differences could be due to the different strains used.

-114 AGTCGGACCGAGTGAGTCAGCGTGATCGGCCCTCA -81

-80 TTTACCTTGTGCGCTTGCACGTCGGTATATAAACGTATGTGGATACTTTAAGAAGGAAAAGCGCAACCGTCCAAATTCTA -1

1 ATG AAC AAA AGA ACA CAG GAA TAC AAG AAC ACA AGA GCG ATA ATG TCT GCC ACT CTG TTC 60
 1 M N K R T O E Y K N T R A I M S A T L F 20

61 AAT AAC ATC GAA TTG CTG CCC CCT GAT GCC CTT TTT GGT ATT AAG CAA AGG TAC GGG CAA 120
 21 N N I E L L P P D A L F G I K Q R Y G Q 40

121 GAT CAA CGT GCT ACC AAG GTC GAC TTG GGT ATC GGG GCC TAC AGA GAC GAC AAC GGT AAA 180
 41 D Q R A T K V D L G I G A Y R D D N G K 60

181 CCA TGG GTC TTG CCA AGT GTT AAA GCC GCC GAA AAG CTA ATT CAT AAC GAC AGC TCC TAC 240
 61 P W V L P S V K A A E K L I H N D S S Y 80

241 AAC CAT GAA TAC CTC GGT ATT ACC GGT CTG CCA AGT TTG ACA TCT AAC GCC GCC AAG ATC 300
 81 N H E Y L G I T G L P S L T S N A A K I 100

301 ATC TTC GGT ACG CAA TCC GAT GCC TTT CAG GAA GAC AGA GTA ATC TCA GTA CAA TCA CTG 360
 101 I F G T Q S D A F Q E D R V I S V Q S L 120

361 TCT GGT ACG GGT GCT CTT CAT ATA TCT GCG AAG TTT TTT TCA AAA TTC TTC CCA GAT AAA 420
 121 S G T G A L H I S A K F F S K F F P D K 140

421 CTG GTC TAT TTG TCT AAG CCT ACT TGG GCC AAC CAC ATG GCC ATT TTT GAG AAT CAA GGC 480
 141 L V Y L S K P T W A N H M A I F E N Q G 160

481 TTG AAA ACG GCG ACT TAC CCT TAC TGG GCC AAC GAA ACT AAG TCT TTG GAC CTA AAC GGC 540
 161 L K T A T Y P W A N E T K S L D L N G 180

541 TTT CTA AAT GCT ATT CAA AAA GCT CCA GAG GGC TCC ATT TTC GTT CTG CAC TCT TGC GCC 600
 181 F L N A I Q K A P E G S I F V L H S C A 200

601 CAT AAC CCA ACT GGT CTG GAC CCT ACT AGT GAA CAA TGG GTT CAA ATC GTT GAT GCT ATC 660
 201 H N P T G L D P T S E Q W V Q I V D A I 220

661 GCC TCA AAA AAC CAC ATC GCC TTA TTT GAC ACC GCC TAC CAA GGG TTT GCC ACT GGA GAT 720
 221 A S K N H I A L F D T A Y Q G F A T G D 240

721 TTG GAC AAG GAT GCC TAT GCT GTG CGT CTA GGT GTG GAG AAG CTT TCA ACG GTC TCT CCC 780
 241 L D K D A Y A V R L G V E K L S T V S P 260

781 GTC TTT GTC TGT CAG TCC TTT GCC AAG AAC GCC GGT ATG TAC GGT GAG CGT GTA GGT TGT 840
 261 V F V C Q S F A K N A G M Y G E R V G C 280

841 TTC CAT CTA GCA CTT ACA AAA CAA GCT CAA AAC AAA ACT ATA AAG CCT GCT GTT ACA TCT 900
 281 F H L A L T K Q A Q N K T I K P A V T S 300

901 CAA TTG GCC AAA ATC ATT CGT AGT GAA GTG TCC AAC CCA CCG GCC TAC GGC GCT AAG ATT 960
 301 Q L A K I I R S E V S N P P A Y G A K I 320

961 GTC GCT AAA CTG TTG GAA ACG CCA GAA TTA ACG GAA CAG TGG CAC AAG GAT ATG GTT ACC 1020
 321 V A K L L E T P E L T E Q W H K D M V T 340

1021 ATG TCC TCC AGA ATT ACG AAA ATG AGG CAC GCA TTA AGA GAC CAT TTA GTC AAG TTG GGC 1080
 341 M S S R I T K M R H A L R D H L V K L G 360

1081 ACT CCT GGC AAC TGG GAT CAT ATA GTA AAT CAA TGC GGG ATG TTC TCC TTT ACA GGG TTG 1140
 361 T P G N W D H I V N Q C G M F S F T G L 380

1141 ACT CCT CAA ATG GTT AAA CGA CTT GAA GAA ACC CAC GCA GTT TAC TTG GTT GCC TCA GGT 1200
 381 T P Q M V K R L E E T H A V Y L V A S G 400

1201 AGA GCT TCT ATT GCT GGA TTG AAT CAA GGA AAC GTG GAA TAC GTG GCT AAA GCC ATT GAT 1260
 401 R A S I A G L N Q G N V E Y V A K A I D 420

1261 GAA GTG GTG CGC TTC TAT GCT ACT GAA GCT AAA TTG TAA TGATTAATAATACTTATACTTACCTAT 1327
 421 E V V R F Y A T E A K L * 433

1328 TACACTCTTCATATTTTTTTTAAATGTAAGAAGTAAAAAATAAAAAAAGAGAAATGCTCTTTCTTACCTT 1407

1408 CACAGGTGATATTATTATGTTGTTATAATATCCGGGTAGGTTTATTGATCCCTCTACGCCGGACGCATCGT 1476

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the AAT2 gene. The C-terminal PTS AKL and the N-terminal sequence before the second start codon are underlined. The amino acids (427 and 428) and bases (positions 942, 1137, 1279 and 1283) that differ from the sequence of AAT2 in the yeast genome project are bold and underlined.

Disruption of the gene for AAT2 leads to a complete loss of peroxisomal AAT activity in oleate-grown cells. To find out whether the gene for AAT2 encodes the peroxisomal AAT activity, we disrupted the AAT2 gene, which led to a strong reduction of total AAT activity, amounting to less than 0.5% of the wild-type level under all growth conditions tested (oleate, glucose, acetate and glycerol; Table 1).

We performed a density-gradient experiment analogous to the one shown in Fig. 1A. When the results of Fig. 1B are com-

pared with those of Fig. 1A, virtually identical profiles of activity were found for the marker enzymes SDH and HAD, whereas the activity profile for AAT is quite different. Although there is not a sharp peak of AAT activity in Fig. 1B, the profile observed for AAT corresponds most closely to that of the mitochondrial marker SDH.

AAT2 import is mediated via the PTS1 receptor. The results obtained above show that the AAT activity encoded by the AAT2

Table 1. Activity of AAT and catalase in *S. cerevisiae* grown on different carbon sources. Cells were grown on oleate, glycerol, acetate and glucose medium. AAT and catalase activity were measured in cell-free extracts and the results of 3–5 experiments are given.

Carbon source	AAT activity in		Catalase activity in wild-type cells
	<i>Aaat2</i> cells	wild-type cells	
	nmol · min ⁻¹ · mg protein ⁻¹		μmol · min ⁻¹ · mg protein ⁻¹
Oleate	1.6 ± 2.1	414 ± 40	744 ± 166
Glycerol	1.9 ± 1.2	365 ± 29	118 ± 15
Acetate	7.0 ± 7.1	663 ± 39	31 ± 6
Glucose	0.7 ± 0.0	314 ± 39	2 ± 1

Table 2. Import of AAT2, PTS1-containing (HAD) and PTS2-containing (thiolase) proteins in control cells, *Δpex7* cells and *Δpex5* cells. Cells were grown on oleate medium and fractionated by differential centrifugation. AAT and HAD activities were measured in the homogenates, organellar pellets and supernatants. The samples were also used for western blotting to determine the amount of thiolase in the fractions. The following recoveries were found: AAT, 83–91%; HAD, 89–119%; thiolase, 93–120%. Results are given as the range observed in 2–5 experiments.

Strain	Activity in organellar fraction of		
	AAT	HAD	thiolase
	%		
Wild-type	65–85	85–95	70–90
<i>Δpex7</i>	65–85	85–95	1–10
<i>Δpex5</i>	10–20	10–20	50–70

gene is peroxisomal under conditions of growth on oleate. To establish whether targeting to peroxisomes is mediated by the putative PTS1 (AKL), we performed a differential-fractionation experiment with three yeast strains: wild-type cells; the *pex7* mutant, which is unable to import PTS2 proteins; and the *pex5* mutant, which is unable to import PTS1 proteins. A marker for the PTS1 route is HAD: it is imported in the *pex7* mutant, but not in the *pex5* mutant. Thiolase is a marker for the PTS2 route: it is imported in the *pex5* mutant, but not in the *pex7* mutant (Marzioch et al., 1994; Zhang et al., 1995; Van der Leij et al., 1993). Inspection of the results of Table 2 shows that AAT activity showed a similar behaviour as HAD, whereas thiolase showed a different pattern. This implies that import of the AAT2 protein is mediated by the PTS1 receptor.

AAT is not induced by oleate. Growth of *S. cerevisiae* on oleate-containing medium leads to a strong induction of peroxisomes and peroxisomal β -oxidation enzyme activities. Furthermore, the peroxisomal MDH (MDH3), which regenerates the NADH produced during fatty acid β -oxidation, is induced by growth on oleate (Van Roermund et al., 1995). To establish whether peroxisomal AAT is induced by growth on oleate, *S. cerevisiae* cells were allowed to grow on different carbon sources, followed by measurement of the AAT activity and catalase activity in cell-free extracts. Table 1 shows that peroxisomes were greatly induced on oleate and repressed on glucose, as concluded from the strongly increased catalase activities. In contrast, AAT activities, as measured in extracts from *S. cerevisiae* grown under different conditions were comparable. There was

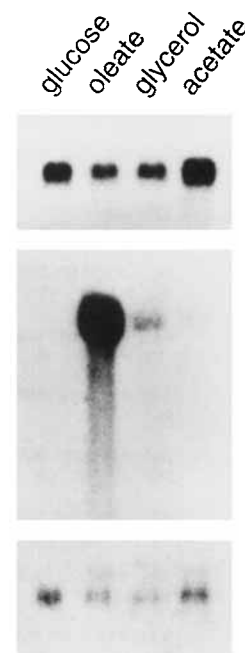


Fig. 4. AAT2 expression levels. RNA of wild-type yeast grown on either rich glucose, oleate, glycerol or acetate was applied to a gel, blotted and incubated with the labeled probes of *AAT2*, *CTAI1* and *PEX13*. *CTAI1* is the control for induction of peroxisomes. *PEX13* is the control for equal amounts of mRNA.

no induction of AAT activity on oleate or repression on glucose, whereas a twofold stimulation could be observed under acetate conditions.

Similar results were obtained upon northern blot analysis with either the *AAT2* probe or the peroxisomal catalase (*CTAI1*) probe. The amount of mRNA of *AAT2* under the different growth conditions was virtually identical, with the exception of acetate-grown cells, in which mRNA levels were slightly higher (Fig. 4). To verify whether equal amounts of mRNA were loaded on the gel, a probe of the peroxisomal assembly gene *PEX13* was used, since this gene is constitutively expressed under the different growth conditions (Elgersma et al., 1996b).

The *aat2*-disruption mutant shows normal growth on oleate.

To establish whether AAT2 functions as part of a malate/aspartate shuttle for reoxidation of intraperoxisomal NADH, we studied growth of the *aat2*-disruption mutant on oleate. Virtually no difference was observed between the growth rate of wild-type cells and the *aat2*-disruption mutant. We also measured the palmitate β -oxidation activity in intact cells. The activity in the *aat2*-disruption mutant and the wild-type cells were comparable (results not shown). These results suggest that AAT2 is not required for the β -oxidation of fatty acids.

The subcellular localization of AAT under different growth conditions.

We found that the activity of AAT was virtually the same irrespective of whether yeast cells were grown on oleate or glucose (Table 1). However, in glucose-grown yeast there are virtually no peroxisomes. To obtain information on the subcellular localization of AAT, cells were fractionated, resulting in an organellar pellet and a soluble cytosolic fraction. The AAT activity was measured in the pellet and the supernatant fractions (Fig. 5A).

When wild-type cells were grown on oleate most of the AAT activity (85%) was found in the organellar fraction, while under

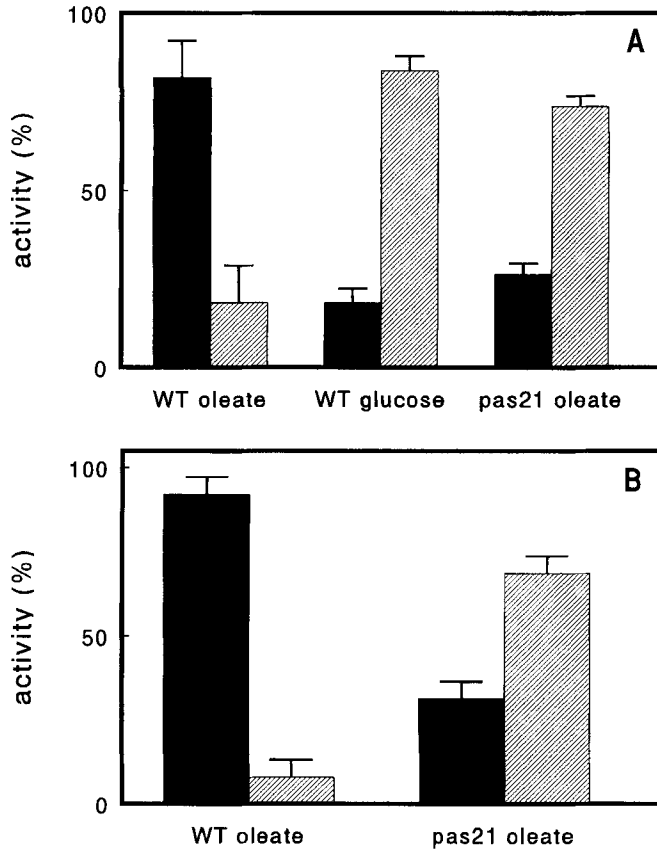


Fig. 5. Localization of AAT activity under different growth conditions. Wild-type (WT) *S. cerevisiae* cells were grown on oleate and glucose medium, and *Apas21* (*pas21*) cells were grown on oleate medium. The cells were fractionated by differential centrifugation and the activities of AAT (A) and HAD (B) were determined in the pellet (black bars) and the supernatant (striped bars). Recoveries of AAT activities were between 83 % and 94 % and recoveries of HAD activities were between 92 % and 116 %.

glucose conditions only 16 % was localized in the organellar fraction (Fig. 5A). HAD and PGI were measured as markers for peroxisomes and cytosol, respectively. Under conditions of growth on oleate, more than 85 % of HAD was located in the organellar pellet (Fig. 5B), whereas more than 90 % of PGI was located in the cytosolic supernatant (results not shown), showing that the method used is satisfactory. HAD activity could not be measured in glucose-grown cells, because of the very low induction on glucose.

As a control, cells of a peroxisome assembly mutant *pas21* (Elgersma et al., 1993) were grown on oleate and fractionated. As expected, the bulk of AAT activity (74 %) was in the supernatant (Fig. 5A).

In the *aat2*-disruption mutant, either grown on oleate or glucose, residual AAT activity is very low, indicating that the contribution of the mitochondrial AAT to total AAT activity is negligible under these conditions. The localization of HAD and PGI in the *aat2*-disruption mutant were the same as in wild-type cells (results not shown).

To establish conclusively that the subcellular localization of AAT is dependent on the growth conditions, we performed additional experiments using digitonin to selectively permeabilize the cell membrane. To this end protoplasts prepared from wild-type cells and *pas21* cells, which were grown under different conditions, were incubated for 10 min in isotonic medium containing increasing amounts of digitonin. After centrifugation the

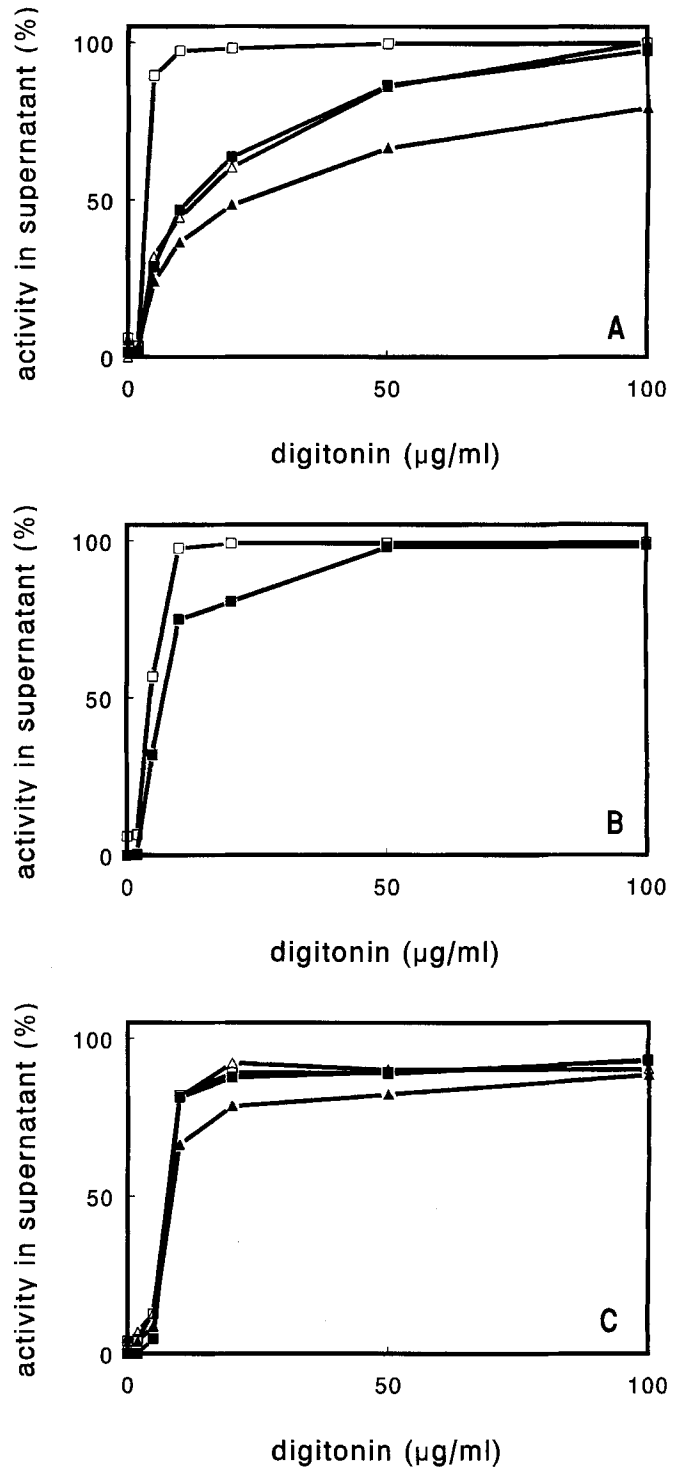


Fig. 6. Release of enzymes from protoplasts treated with different concentrations of digitonin. Wild-type (WT) cells were grown on oleate and glucose, and *Apas21* (*pas21*) cells were grown on oleate medium. Protoplasts were treated with different concentrations of digitonin, and the enzyme release was measured in the obtained supernatant fractions (□, PGI; ■, AAT; △, catalase; ▲, HAD). Maximal activity was obtained with 0.1 % (mass/vol.) Triton X-100. (A) Wild-type cells grown on rich oleate medium; (B) wild-type cells grown on rich glucose medium; (C) *Apas21* cells grown on rich oleate medium.

activities of PGI, AAT, catalase and HAD were measured in the pellets and supernatants. The cytosolic enzyme PGI was released at low digitonin concentrations independent of whether control cells (Fig. 6A and B) or peroxisome-deficient cells (*Apas21*;

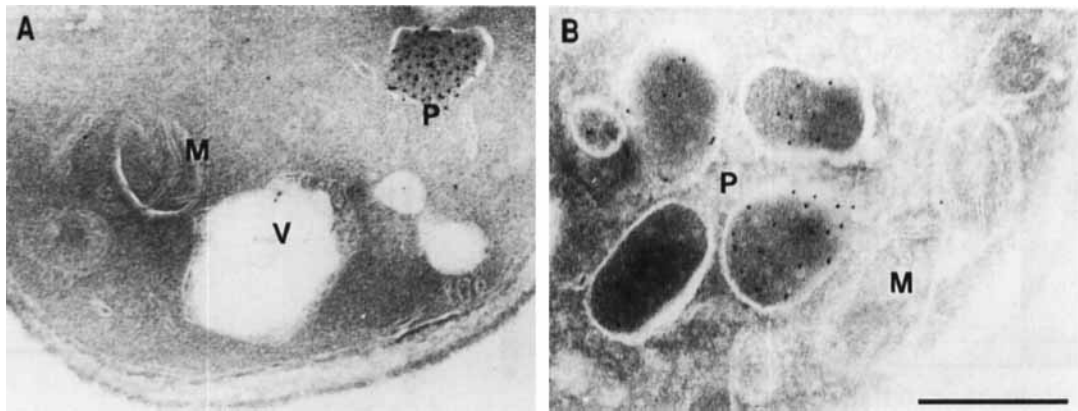


Fig. 7. Electron-microscopic analysis of the intracellular localization of MDH3 under oleate and glucose conditions. Glucose-grown (A) and oleate-grown (B) cells, expressing tagged MDH3 under the control of the PGK promoter, were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Ultrathin sections were prepared and incubated with the NH antibody. Bar = 0.5 μ m. M, mitochondrion; V, vacuole; P, peroxisome.

Table 3. Subcellular localization of the long and short form of AAT2.

The *aat2*-disruption mutant with a single-copy plasmid with the long construct (pAAT2long) or short construct (pAAT2short) from AAT2 was grown on oleate and glucose medium and fractionated by differential centrifugation. In the fractions the AAT activity was measured. The recoveries varied between 78% and 91%. The average of three separate colonies of each strain are given.

Strain	Medium	AAT activity in organellar fraction
		%
<i>Aaat2/pAAT2long</i>	oleate	71.5 \pm 6.1
	glucose	13.7 \pm 3.8
<i>Aaat2/pAAT2short</i>	oleate	77.1 \pm 9.7
	glucose	13.4 \pm 1.8

Fig. 6C) were used. Release of the peroxisomal enzymes catalase and HAD was achieved at much higher digitonin concentrations (Fig. 6A). AAT shows a pattern of release comparable to those of catalase and HAD, suggesting a peroxisomal localization of AAT in these oleate-grown cells.

In peroxisome-deficient cells catalase, HAD and AAT show a similar pattern of release as PGI, showing that all these enzymes are cytosolic in peroxisome-deficient cells (Fig. 6C).

When the pattern of release of AAT in glucose grown cells is considered (Fig. 6B), it is clear that at low digitonin concentrations 75% of AAT2 was released from the cells, indicating that under glucose conditions AAT2 is mainly localized in the cytosol.

The PTS1 import pathway is functional in glucose-grown cells. The apparent cytosolic localization of AAT2 in glucose-grown cells may be explained by the lack of an active PTS1 import machinery under these conditions. To test whether this is the case we expressed a haemagglutinin-tagged PTS1-reporter protein (MDH3) (Elgersma et al., 1996a) under the control of the constitutive PGK promoter. Cells expressing tagged MDH3 were grown on oleate and glucose medium and fractionated by differential centrifugation. We found that under both conditions tagged MDH3 was predominantly (85%) located in the organellar pellet fraction (data not shown). To verify whether this was not caused by aggregation of the protein due to the strong promoter used, we examined the MDH3-expressing cells by electron microscopy. Tagged MDH3 was present in peroxisomes

in glucose-grown and oleate-grown cells (Fig. 7). In addition we conclude from these experiments that glucose-grown cells can be used for subcellular fractionation of peroxisomes.

AAT2 has two possible start sites. Sequencing of the AAT2 gene revealed the existence of two possible start sites (Fig. 3) at amino acid positions 1 and 15. Normally, transcription starts at the first start codon (ATG). However, Cronin et al. (1991) determined the amino acid sequence of AAT2 under glucose conditions, and they found that transcription of the AAT2 gene starts at the second start codon.

To establish whether the differential targeting observed for AAT2 upon grown on glucose and oleate could be explained by the existence of two transcription products (as has been shown for carnitine acetyltransferase; Elgersma et al., 1995), we constructed two fragments: a long fragment containing the whole AAT2 gene, and a short fragment containing the AAT2 gene starting from amino acid 15. The fragments were cloned in a single-copy plasmid, behind a constitutive promoter, and transformed to the *aat2*-disruption mutant. These cells were grown on oleate and glucose medium, followed by differential centrifugation. AAT was predominantly present in the organellar pellet in oleate-grown yeast cells, irrespective of whether cells were transformed with the long or the short construct (Table 3). When the same cells were grown on glucose, most of the AAT activity was recovered in the supernatant. These results indicate that the two putative translation initiation sites are not important for determining the subcellular localization of AAT2.

DISCUSSION

We recently presented evidence suggesting that peroxisomal MDH (MDH3) is involved in the intraperoxisomal reoxidation of NADH, possibly as part of a malate/aspartate shuttle. If operative in peroxisomes, such a shuttle would require the presence of AAT activity in peroxisomes and cytosol.

In the literature no mention is made of a peroxisomal AAT in *S. cerevisiae*. It was generally assumed that there were only two AAT, one in the cytosol (Cronin et al., 1991) and one in mitochondria (Morin et al., 1992). The results described in this paper show that there is AAT activity in peroxisomes. Our data show that peroxisomal and cytosolic AAT are the products of a single gene (AAT2) since a disruption mutant is deficient in cytosolic and peroxisomal AAT. Cronin and coworkers found that the C terminus of 'cytosolic' AAT ends in the tripeptide Ala-

Lys-Leu, which has been shown to function as a PTS (McNew and Goodman, 1994; Elgersma et al., 1995).

The total cellular AAT activity is almost comparable under the different growth conditions, whereas the distribution of AAT between cytosol and peroxisomes is markedly different under oleate and glucose growth conditions. In glucose-grown cells most of the activity is localized in the cytosol, whereas in oleate-grown cells the bulk of activity is found in the peroxisomes. This observation is puzzling since MDH3, another peroxisomal enzyme with a PTS1-dependent targeting signal (SKL), is localized in peroxisomes under oleate and glucose conditions. There are several options to explain the difference in localization of the two peroxisomal enzymes. Firstly, AAT2 may possess an extra signal to direct AAT2 to peroxisomes under oleate conditions, or secondly, AAT2 may have an extra signal to retain AAT2 in the cytosol under glucose conditions. We investigated the involvement of the N terminus of AAT2 in targeting, because AAT2 has two possible starting positions. Under oleate conditions another transcript could be present than under glucose conditions. However, our results show that the N terminus does not contain the extra signal that is necessary for differential targeting.

The *aat2*-disruption mutant did not grow on minimal glucose medium. We found out that this was due to an aspartate deficiency. This indicates that at least under glucose conditions, where AAT2 is mainly localized in the cytosol, AAT2 is involved in the biosynthesis of aspartate.

Despite the presence of high AAT activity in peroxisomes under oleate conditions, the results described indicate that AAT2 is not essential for intraperoxisomal reoxidation of NADH to NAD. Firstly, AAT2 is not induced on oleate, which suggests that this enzyme is not involved in the β -oxidation process. Secondly, the *aat2*-disruption mutant shows normal growth on oleate. Thirdly, intact cells of the *aat2*-disruption mutant showed normal β -oxidation, indicating that AAT2 plays a dispensable role in peroxisomal fatty acid β -oxidation.

In contrast, MDH3, which is involved in the peroxisomal redox shuttle, is induced by oleate and deletion of the gene results in impaired growth on oleate and in a block in the β -oxidation (Van Roermund et al., 1995). If AAT2 is not required in a malate/aspartate shuttle, the possibility should be considered that oxaloacetate (which is a substrate for MDH3) is transported across the peroxisomal membrane. This would not be unprecedented, since it has been proposed that oxaloacetate can traverse the inner membrane of mitochondria (Douce and Bonner, 1972), a process which is mediated by the dicarboxylate carrier (Gimpel et al., 1973). Cloning of the peroxisomal carriers will provide further insight into how reducing equivalents are shuttled across the peroxisomal membrane.

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