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

Verleur, N.; Elgersma, Y.; van Roermund, C.W.T.; Tabak, H.F.; Wanders, R.J.A.

Published in:
European Journal of Biochemistry

DOI:
10.1111/j.1432-1033.1997.00972.x

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)

Download date: 07 Sep 2020
Cytosolic aspartate aminotransferase encoded by the AAT2 gene is targeted to the peroxisomes in oleate-grown Saccharomyces cerevisiae

Nicolette VERLEUR1, Ype ELGERSMA2, Carlo W. T. VAN ROERMUND1, Henk F. TABAK2 and Ronald J. A. WANDERS1

1 Department of Clinical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands
2 Department of Biochemistry, E.C. Slater Institute, Academic Medical Center, Amsterdam, The Netherlands

(Received 12 February 1997) -- EJB 97 0230/1

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, 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 re-action 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, trpl, ura3-251, prb1-1122, pep4-3) (as wild-type strain for gene disruptions and gradients) and pas21 (PAS21::LEU2, MATa, leu2, trpl, ura3-251, prb1-1122, pep4-3) and pex13 (PEX13::LEU2, MATa, leu2, trpl, ura3-251, prb1-1122, pep4-3) (as control strains without functional peroxisomes). The Escherichia coli strain used for cloning procedures was DH5αF’ (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 μg/ml L-tryptophan, 20 μg/ml uracil,
30 μg/ml l-leucine) or on rich galactose medium (medium B) containing 2% galactose, 1% yeast extract and 2% peptone. Rich yeast media 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 or oleate medium. The cultures were inoculated at such an A660 that after incubation for 15 h an A660 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)A(T/C)AA(A/G)CC(G/G/A/T/I)CTGGGTT-3' (based on the amino acid sequence DDNGKPWV) and 5'-ACCAT(A/G)TT(C/T)TTA(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 HindIII site of pUC19 (resulting in pEL74) and sequenced. To construct a disruption plasmid, the EcoRI/SphI fragment of pEL74 was sub-cloned 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 Tin5 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 GITLPSL) and 5'-CGTTAATTCTG-GCGTTTCCAAC-3' (based on the amino acid sequence TLEPCCATGAACAAAAGAACA~AGGAATACAAGAAC-3'). The obtained product was used to transform the wild-type strain BJ1991. Transformed cells were grown on medium B, and incubated with 20-100 μg/ml phleomycin. The transformants were rescued on minimal glucose medium 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 AAT2 gene (AAT2) from a yeast genomic library in the multicycopy 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 Maccecchi (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 (CTAI) 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 fractions 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 19,000 rpm (29,000 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) supplemented 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. Olate-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 13,000 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.
RESULTS

Identification of AAT in peroxisomes. To investigate the involvement of AAT in a peroxisomal redox shuttle, we studied 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 AAT 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 were Cronin and colleagues described two unknown amino acids on positions 232 and 233 (Fig. 3). These differences could be due to the different strains used.
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 compared 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.

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 the wild-type gene, which led to a strong reduction of AAT activity, are bold and underlined.

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

coded the peroxisomal AAT activity in oleate-grown cells. Disruption of the gene for AATZ leads to a complete loss of acetate and glycerol; Table 1).
AAT is not induced by oleate. Growth of S. cerevisiae on oleate-containing medium leads to a strong induction of peroxisomes and peroxisomal \( \beta \)-oxidation enzyme activities. Furthermore, the peroxisomal MDH (MDH3), which regenerates the NADH produced during fatty acid \( \beta \)-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 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 (CTA1) 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 \( \beta \)-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 \( \beta \)-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 *Apa21* (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%.

Throughout glucose conditions only 16% was localized in the organelar 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 organelar 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 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 (*Apa21*;
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. We found that under both conditions 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 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. Second, 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 mitochondrion 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 (Gimper et al., 1973). Cloning of the peroxisomal carriers will provide further insight into how reducing equivalents are shuttled across the peroxisomal membrane.

We would like to thank Marlene van den Berg for the electron microscopy work.

REFERENCES


Verleur et al. (Eur. J. Biochem. 247)


