THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 42, Issue of October 18, pp. 26375–26382, 1996
Printed in U.S.A.

Analysis of the Carboxyl-terminal Peroxisomal Targeting Signal 1 in a Homologous Context in *Saccharomyces cerevisiae**

(Received for publication, June 24, 1996, and in revised form, August 1, 1996)

Ype Elgersma‡§, Arnold Vos‡¶, Marlene van den Berg‡, Carlo W. T. van Roermund‖, Peter van der Sluijs, Ben Distel‡, and Henk F. Tabak‡**

*From the ‡Department of Biochemistry, Academic Medical Centre, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands, the ‖Departments of Pediatrics and Clinical Biochemistry, Academic Medical Centre, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands, and the **Department of Cell Biology, Utrecht University School of Medicine, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*

Most peroxisomal matrix proteins contain a carboxyl-terminal tripeptide that directs them to peroxisomes. Within limits, these amino acids may be varied, without loss of function. The specificity of this peroxisomal targeting signal (PTS1) is remarkable considering its small size and its relaxed consensus sequence. Moreover, several peroxisomal proteins have a PTS1-like signal that does not fit the reported consensus sequence. Because many of these PTS1 variants seem to be functional in a species-dependent or protein context-dependent manner, we investigated the PTS1 requirements in a homologous context, using *Saccharomyces cerevisiae* and endogenous peroxisomal malate dehydrogenase (MDH3). Peroxisomal import of the MDH3-PTS1 variants was tested qualitatively by the ability to complement the Δ*mdh3* mutant and quantitatively by subcellular fractionation. We observed efficient import of MDH3 into peroxisomes with a large variety of PTS1 tripeptides. Many of these variants do not fit the observed PTS1 requirements for heterologously expressed proteins, which suggests that additional domains in the protein may be of decisive importance whether or not a certain PTS1 variant is recognized by the components of the peroxisomal import machinery. Because we show that dimerization of MDH3 precedes import into the organelle, these domains are most likely conformational domains.

Peroxisomes are nearly ubiquitous organelles bounded by a single membrane. Their function differs from organism to organism but always includes the β-oxidation of fatty acids. Peroxisomal matrix proteins are synthesized in the cytoplasm and are targeted to peroxisomes by virtue of a peroxisomal targeting signal (PTS).[1] Two types of peroxisomal targeting signals have been identified so far. The PTS1 is located at the extreme COOH terminus of a protein and was first identified in firefly luciferase, which is targeted to peroxisomes when expressed in mammalian cells (1, 2). The PTS1 signal is present in the majority of the peroxisomal matrix proteins. The second peroxisomal targeting signal (PTS2) resides at the NH_2 terminus of a protein and was first identified in thiolase (1–4). Only a limited number of peroxisomal matrix proteins are imported via this signal.

The PTS1 is a remarkable targeting signal in two aspects. First, the PTS1 is very small, because it consists of only a tripeptide. Second, considering the length of the targeting signal, the PTS1 consensus sequence is relaxed. Mutational analysis of the tripeptide of luciferase resulted in the following consensus sequence: (S/A/C)(K/H/R)(L/M) (in one-letter amino acid notation) or more general: a small amino acid at the first position, a basic amino acid at the second position, and a leucine (or methionine, although this was less efficient) at the last position. These conclusions were based on an immunofluorescence import assay in transfected mammalian cells expressing the heterologous luciferase gene or hybrid genes of chloramphenicol acetyltransferase fused to luciferase (5, 6). An even more relaxed consensus PTS1 was observed for import of proteins into glycosomes of trypanosomes (7, 8). For these studies either luciferase was used or a hybrid protein of the COOH-terminal 6 amino acids of glycosomal phosphoglycerate kinase fused to chloramphenicol acetyltransferase. Import was determined using a selective permeabilization assay.

Recent observations justified reinvestigation of the nature of the PTS1. First, PTS1-resembling sequences have now been identified in a large number of proteins (reviewed by Refs. 9–11), and this number is rapidly increasing by the cloning of genes encoding peroxisomal proteins. Although the consensus PTS1 originally seemed to be a simple one, more and more PTS1-like sequences are identified that do not match the originally defined consensus sequence. Because these PTS1 variants were mostly found in trypanosomes and yeasts, this has been explained by species divergence. However, the recent observation that a human protein (alanine:glyoxylate aminotransferase) can also be targeted to peroxisomes of mammalian cells by PTS1 tripeptides (KKL, SSL, SQL, and NKL) that do not fit the consensus sequence makes the proposed species divergence questionable (12). A second reason to re-examine the nature of PTS1-dependent import is the remarkable finding that peroxisomes are able to import folded proteins (13–15). These findings may indicate that the folded structure of the protein is of decisive importance for recognition of the PTS1. All comprehensive mutation analyses of the PTS1 have been performed with either heterologously expressed peroxisomal proteins or with nonperoxisomal reporter proteins, without check on the functional competence of the imported protein (5–8). Therefore we decided to analyze the PTS1 signal in the homologous context and in a more quantitative and functional manner. To this purpose, we have made mutations in the PTS1 signal of the endogenous MDH3 protein of *Saccharomyces cer-*

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Present address: University of California, San Diego, Dept. of Biology, 9500 Gilman Dr., La Jolla, CA 92093-0322.

¶ Present address: Netherlands Cancer Inst., Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

[1] The abbreviations used are: PTS, peroxisomal targeting signal; PCR, polymerase chain reaction; CHAPS, 3[(cholamidopropyl)dimethyl-ammonio]-1 propanesulfonate; MDH, malate dehydrogenase.

evisiae. Import of the MDH3-PTS1 mutants was qualitatively scored by the (in)ability to complement the Δ*mdh3* mutant and was quantified by subcellular fractionation. We conclude that a wide variety of PTS1 signals are functional when presented in a homologous context and discuss the implications for the interaction between the PTS1-containing protein and the PTS1 receptor.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—Yeast strains used for this study were the *S. cerevisiae* strains BJ1991 (*MAT a, leu2, trp1, ura3–251, prb1–1122, pep4–3*) and Δ*mdh3* (*MDH3::LEU2*) and Δ*pex5* (Δ*pas10*) (*PEX5::LEU2*), which were made in BJ1991 as described in Refs. 16 and 17. The *pex7* (*pas7*) mutant is described in Ref. 18.

Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and amino acids (20 μg/ml) as needed. Liquid oleate medium used for induction contained 0.5% potassium phosphate buffer (pH 6.0), 0.3% yeast extract (Difco), 0.5% peptone, and 0.12% oleic acid/0.2% Tween 40 as carbon source. Before shifting to this medium, cells were grown on 0.3% glucose minimal medium for at least 24 h. Oleic acid plates contained 0.1% oleic acid/0.4% Tween 40, 0.67% yeast nitrogen base without amino acids, 0.1% yeast extract, and amino acids (20 μg/ml) as needed. For immunoprecipitations, we used cells grown on minimal medium containing 0.67% yeast nitrogen base without amino acids, amino acids (20 μg/ml) as needed and either 2% glycerol or 0.1% oleic acid/0.4% Tween 40.

Western Blotting—Proteins were separated on 12% SDS-polyacrylamide gel electrophoresis gels and transferred to nitro-cellulose paper in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). For qualitative analysis, the blots were blocked overnight by incubation in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20) supplied with 2% nonfat dry milk. The same buffer was also used for incubation with the primary antibody and for IgG-coupled alkaline phosphatase. The blots were stained in AP buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$) with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as per the manufacturer's instructions (Boehringer Mannheim). For quantitative analysis, blots were blocked in phosphate-buffered saline with 0.5% gelatin, 0.5% Tween 20. For incubation with antibodies, we used phosphate-buffered saline with 0.2% gelatin, 0.5% Tween 20. Blots were washed in phosphate-buffered saline with 0.2% gelatin, 0.5% Tween 20 and incubated with ^{125}I-labeled protein A followed by quantitation using a PhosphorImager (Molecular Dynamics).

NH Epitope Tagging and Antibodies—The synthetic NH epitope tag CQDLPGNDNST (corresponding to the NH_2 terminus of the mature hem-agglutinin protein) was conjugated with maleimide bis *N*-hydroxysuccinimide to keyhole limpet hemocyanin and used for antibody production in rabbits. For epitope tagging, an oligonucleotide adaptor encoding the NH epitope

```
          M  Q  D  L  P  G  N  D  N  S  T  A  G  G  T

5′-CACCATGCAAGACCTTCCAGGAAATGACAACAGCACAGCAGGTG-3′

3′-TCGAGTGGTACGTTCTGGAAGGTCCTTTACTGTTGTCGTGTCGTCCACCTAG-5′
```

was ligated in the *Sac*I/*Bam*HI site of the CTA1 expression plasmids. People interested in these epitope tagging reagents should contact P. van der Sluijs. The raising of thiolase antibodies is described in Ref. 19.

Construction of MDH3-SKL/SEL/ΔKL Mutants—pMDH3-SEL was obtained by PCR on genomic DNA of *S. cerevisiae* using the primers 5′-AAGGATCCATGGTCAAAGTCGCAATTCTTG-3′ and 5′-AAAAAGCTTCATAGCTCGGAAGAGTCTACGATGAAACTC-3′. After digestion of the created *Bam*HI and *Hin*dIII sites, the fragment was cloned into the *Bam*HI/*Hin*dIII site of pUC19 (pUC-MDH3-SEL). MDH3-SKL was made by replacing the *Cla*I/*Hin*dIII fragment of pUC-MDH3-SEL by the *Cla*I/*Hin*dIII fragment of pUC-MDH3 (16) resulting in pUC-MDH3-SKL. The MDHΔKL gene was obtained by PCR on genomic DNA of *S. cerevisiae*, using the primers 5′-AAGGATCCATGGTCAAAGTCGCAATTCTTG-3′ and 5′-AAAAAGCTTCATAGCTAGGAAGAGTCTACGATGAAACTC-3′. This fragment was cut with *Cla*I/*Hin*dIII and cloned into the *Cla*I/*Hin*dIII site of pUC-MDH3-SEL resulting in pUC-MDH3ΔKL. All constructs were verified by sequencing starting from the 3′ end until the *Cla*I site to make sure that all genes were identical except for the PTS1. The genes were subsequently cloned in the *Bam*HI/*Hin*dIII site behind the catalase promoter of pEL43 (20), which is derived from pYCplac33 (21). The multicopy plasmid used was similar to pEL43 except that it was based on pYEplac 181 (21).

```
                I   L   D   S   S   K   L   *
MDH3-SKL        ACC CTA GAC TCT TCC AAG CTA TGA

                I   L   D   S   S   E   L   *
MDH3-SEL        ACC CTA GAC TCT TCC GAG CTA TGA

                I   L   D   S   S   *
MDH3ΔKL         ACC CTA GAC TCT TCC TAG

                I   L   E   S   T   C   R   H   A   S   L   S   N   K   *
MDH3ΔPTS1       ACT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTA AGT AAC AAA TGA
                    XbaI            PstI

                I   L   D   S   X   K   L   *
MDH3-XKL        ACT CTA GAC TCC NNS AAG CTA TGA CTG CAG GCA
                TGA GAT CTG AGG NNS TTC GAT ACT GAC GTC CGT

                I   L   D   S   S   X   L   *
MDH3-SXL        ACT CTA GAC TCC TCC NNS CTA TGA CTG CAG GCA
                TGA GAT CTG AGG AGG NNS GAT ACT GAC GTC CGT

                I   L   D   S   S   K   X   *
MDH3-SKX        ACT CTA GAC TCC TCC AAG NNS TGA GCG CTG CAG
                TGA GAT CTG AGG AGG TTC NNS ACT CGC GAC GTC
```

FIG. 1. **MDH3 constructs used in this study.** The degenerated adaptors used for random mutagenesis are printed in *bold*. *X* represents any amino acid. The nucleotide code *N* represents any base, whereas the nucleotide code *S* represents a C or G.

Construction of MDH3-PTS1 Mutant Library—To construct a library of MDH3-PTS1 mutants, an *Xba*I restriction site was created by introducing a silent mutation 19 base pairs upstream of the stopcodon of the MDH3 gene. This was achieved by PCR on pWT-MDH3 (pEL102) using a universal primer and the MDH3-Xba primer 5′-GAAGAGTCTAGAATGAAACTCTTGCG-3′. The obtained PCR fragment was digested with *Bam*HI and *Xba*I and cloned behind the CTAI promoter of pEL43 (20). To exclude PCR mistakes, this construct was sequenced up to the *Cla*I site, and the remainder of the gene was replaced with the *Sac*I-*Cla*I fragment of pNH-MDH3-SKL (pEL143), resulting in NH-MDH3ΔPTS1 (pEL149). This plasmid was cut with *Xba*I and *Pst*I, purified, and recirculated by ligating in the presence of the degenerated oligonucleotide adaptors (Fig. 1). The ligation mixture was transformed to a *Escherichia coli* MutS strain. The obtained colonies were rinsed from the plates, and plasmid DNA was isolated. This DNA was retransformed to *E. coli* (DH5α), and DNA was isolated from single colonies. This was sequenced from the 3′ end up to approximately 20 base pairs after the *Xba*I site. Although we often found unexpected mutations at the place of the introduced oligos, we never found mutations before the *Xba*I site. We had to sequence about 70 clones to obtain the reported random mutations in the tripeptide. The FFF and ANL tripeptides were made by using nondegenerated adaptors. After sequencing, the selected plasmids were tranformed to the Δ*mdh3* mutant.

Electron Microscopy—Oleate-induced cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Ultra-thin sections were prepared as described in Ref. 22.

Subcellular Fractionation and Nycodenz Gradients—Subcellular fractionations and Nycodenz gradients were performed as described previously (23).

Enzyme Assays—Enzymes were measured as described earlier (24).

Immunoprecipitations—20 ml of a culture of cells grown overnight on 0.3% glucose minimal medium was spun, and the cells were resuspended in 5 ml of minimal glycerol or oleate medium. After growing the cells for another 2 h, the cells were collected and resuspended in 1 ml of minimal glycerol medium or oleate medium with 25 μCi of ^{35}S-labeled methionine and cysteine. After an incubation of 1.5 h (28 °C, shaking), cells were transferred in 2-ml Eppendorf tubes, spun, and resuspended in immunoprecipitation buffer (50 mM Tris (pH 7.5), 50 mM NaCl, 0.2% Triton X-100). After adding glass beads, the cells were lysed by vortexing for 30 min at 4 °C. The cell debris and glassbeads were removed by a short spin. Subsequently, 100 μl of a 10% protein A beads suspension (washed in HBS buffer (50 mM HEPES-NaOH (pH 7.6), 200 mM NaCl) containing 0.5% CHAPS and 2.5 μl of NH-antibody were added to the cell-free lysate, and this was incubated for 1 h at 4 °C while shaking. The beads were collected by a short spin, washed two times with immunoprecipitation buffer, and heated in Laemmli sample buffer. Beads were spun down, and the supernatants were then layered on a 15% SDS-polyacrylamide gel. After staining and destaining, the gels were washed in 30% methanol/phosphate-buffered saline for 5 min and then incubated in enhancer (20 g/liter of sodium salicylate in 30% methanol), dried, and subjected to fluorography.

The glycerol medium appeared to be especially useful for labeling

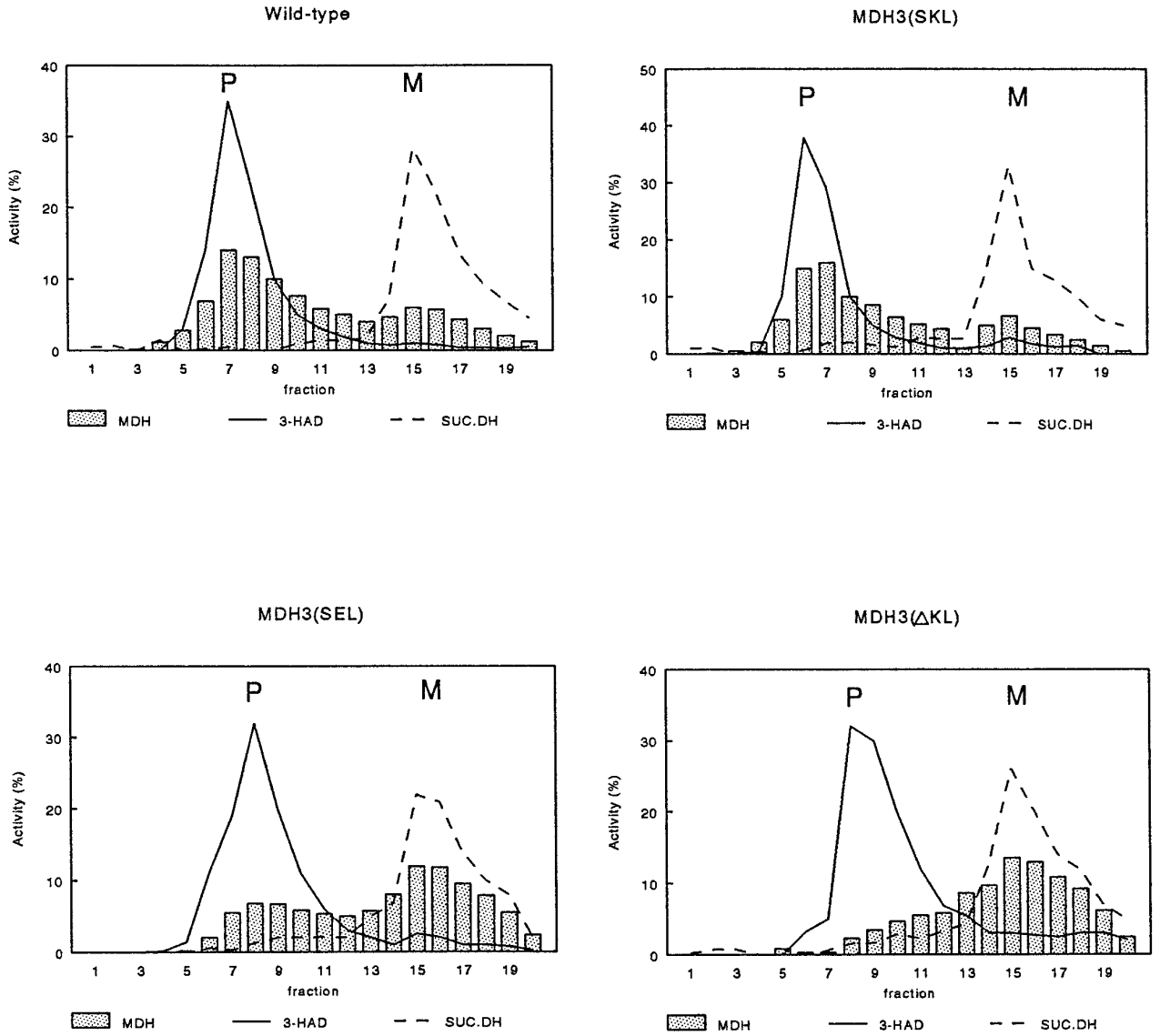

FIG. 2. **Subcellular localization of malate dehydrogenase in *S. cerevisiae*.** An organellar pellet was obtained by subcellular fractionation of cells induced on oleate and used for density gradient centrifugation on Nycodenz. Fraction 1 represents the bottom fraction, and fraction 20 represents the top fraction. Succinate dehydrogenase and 3-hydroxy acyl-CoA dehydrogenase were measured as mitochondrial and peroxisomal markers, respectively. *A*, wild-type cells. *B*, Δ*mdh3* + pMDH3-SKL. *C*, Δ*mdh3* + pMDH3-SEL. *D*, Δ*mdh3* + pMDH3ΔKL.

experiments with cells that are unable to grow on oleate. For wild-type cells, the results were identical for the two media used.

RESULTS

MDH3 Import Is PTS1-dependent—Peroxisomal malate dehydrogenase of *S. cerevisiae* (MDH3) possesses the typical PTS1 tripeptide SKL at its COOH terminus (25). Because some PTS1-containing proteins contain additional (internal) peroxisomal targeting signals (23, 26, 27), we tested whether import of MDH3 completely relies on this tripeptide. To this purpose we made one PTS1 mutant from which the last two amino acids were deleted (MDH3ΔKL) and one PTS1 mutant in which the positively charged lysine residue was replaced by the negatively charged glutamic acid residue (MDH3-SEL). Previously, it has been shown that this substitution blocks peroxisomal import of luciferase in CV-1 cells and in *S. cerevisiae* (5, 20, 28). The wild-type gene (MDH3-SKL) and the mutant genes were placed under the control of the peroxisomal catalase promoter (CTA1) and transformed to Δ*mdh3* cells. Recently, we demonstrated that Δ*mdh3* cells were unable to grow on plates containing oleate as sole carbon source (16). Import of the MDH3-PTS1 variants was tested by the ability of the transformants to complement the growth defect of Δ*mdh3* cells. Δ*mdh3* cells transformed with MDH3-SKL regained their ability to grow on oleate plates. No growth was observed in Δ*mdh3* cells transformed with the MDH3ΔKL plasmid, indicating that the SKL tripeptide is required for import of MDH3 into peroxisomes and that no additional peroxisomal targeting signals are present in this protein (data not shown). Surprisingly, we found that Δ*mdh3* cells expressing MDH3-SEL fully regained the ability to grow on oleate. This indicates that sufficient amounts of MDH3-SEL were imported into peroxisomes to give functional complementation.

To measure the efficiency of import, we determined the amount of MDH3 in peroxisomes. Wild-type cells and Δ*mdh3* transformants were induced on oleate, followed by subcellular fractionation. To eliminate the contributions of cytosolic and mitochondrial malate dehydrogenase (MDH2 and MDH1, respectively), the organellar pellet was further fractionated on a Nycodenz density gradient. Import into peroxisomes was roughly estimated by determining the distribution of MDH activity between the peroxisomal and the mitochondrial peak fractions (*P* and *M*, Fig. 2). The MDH distribution in wild-type

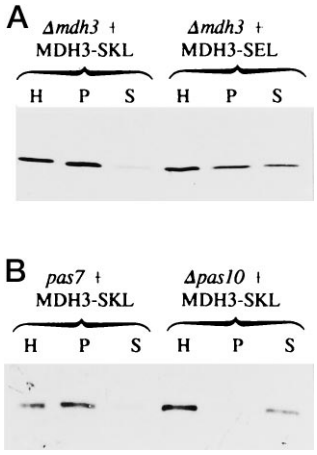

FIG. 3. **Western blot showing the subcellular distribution of the NH-MDH3 proteins.** *A*, Δ*mdh3* cells expressing MDH3-SKL or MDH3-SEL. *B*, NH-MDH3-SKL expressed in *pex7* (*pas7*) cells or in Δ*pex5* (Δ*pas10*) cells. The 30,000 × *g* pellet fraction (*P*) represents the organellar fraction, whereas the supernatant (*S*) fraction represents the cytosolic fraction. *H* represents the homogenate before the high speed spin. An equal percentage was layered in every lane. Pelletable thiolase was at least 75% for Δ*pex5* (Δ*pas10*) and less than 10% for *pex7* (*pas7*). For this figure, the proteins were detected using NH antibodies and an alkaline phosphatase-based color staining.

cells was similar to the distribution observed in Δ*mdh3* cells transformed with pMDH3-SKL, indicating that the output of the endogenous MDH3 promoter is very similar to the output of the CTA1 promoter under these conditions. No bimodal MDH distribution was observed in Δ*mdh3* cells transformed with pMDH3ΔKL, which is in line with the observation that this plasmid was not able to complement the Δ*mdh3* mutant. Approximately 30% of MDH3-SEL was imported into peroxisomes, based on the P:M ratio of MDH3-SEL (P:M = 0.6:1) compared with that of MDH3-SKL (P:M = 2:1).

Epitope Tagging of the MDH3 Protein—To determine the import of the MDH3-PTS1 variants in a more convenient and quantitative manner, we epitope-tagged the protein at its NH_2 terminus with the NH tag. After subcellular fractionation of the transformants, the amount of NH-MDH3 in the organellar pellet fraction and cytosolic supernatant fraction was determined by Western blotting using the antibody against the NH epitope, followed by ^{125}I-labeled protein A. The amount of ^{125}I labeling was quantified with a PhosphorImager. At least 90% of NH-MDH3-SKL proved to be present in the organellar pellet (Fig. 3*A*). No import was observed for the NH-MDH3ΔKL protein (data not shown), whereas we found that on average 50% of the NH-MDH3-SEL protein was imported into peroxisomes (Fig. 3*A*). These results are in agreement with the rough estimates based on the gradient analysis, indicating that the NH tag did not interfere with targeting of MDH3.

Import of NH-MDH3-SEL into peroxisomes could also be demonstrated with immunoelectronmicroscopy. As shown in Fig. 4*B*, NH-MDH3-SEL was present inside the organelle and not just associated with it. This is consistent with the observation that (NH-)MDH3-SEL complemented the growth defect of Δ*mdh3* cells. No import was observed in Δ*mdh3* cells expressing NH-MDH3ΔKL (not shown).The tagged protein also enabled us to study the import of MDH3 in the PTS1 import mutant, *pex5* (formerly named *pas10*), and the PTS2-import mutant, *pex7* (formerly named *pas7*) (17, 29, 30). Import of NH-MDH3-SKL was normal in *pex7* cells but blocked in *pex5* cells, indicating that import of this protein into peroxisomes is dependent on the presence of the PTS1 receptor (Fig. 3*B*).

Import of MDH3-PTS1 Variants—It was remarkable that MDH3-SEL was imported into peroxisomes, because it has previously been demonstrated that luciferase-SEL or bleomycin-luciferase-SEL cannot be imported into peroxisomes of *S. cerevisiae* (20, 28). This suggests that some targeting signals are functional in endogenous peroxisomal proteins but not in the context of heterologously expressed proteins. To test this hypothesis, we constructed MDH3-PTS1 variants with substitutions at the first, second, and third position of the tripeptide. The resulting constructs were sequenced and transformed to Δ*mdh3* cells. Import was determined by the ability of the transformants to grow on oleate and by subcellular fractionation followed by Western blotting and quantitation using a PhosphorImager (Table I).

We could not find any amino acid substitution at the first position of the tripeptide that had an influence on the import efficiency. Many substitutions were also tolerated at the second position of the tripeptide. The most severe mutations obtained at this position were the negatively charged glutamic acid (E) and the small alanine (A) residue, but even the majority of these MDH3-PTS1 variants were imported. Furthermore, we found that positively charged amino acids (K/R/H) could be replaced by aromatic amino acids (Y/F) without any noticeable effect on the import efficiency. The number of functional mutations at the last position of the tripeptide was much more limited, as has also been observed in the luciferase import studies (5). MDH3-PTS1 variants which were imported for only 5% (SKN and SKE), were still able to complement the Δ*mdh3* mutant. No complementation (and import) was observed when the ultimate amino acid was replaced by the charged residues arginine (R) or aspartic acid (D). Because these proteins were able to complement the growth defect of a Δ*mdh2* mutant on ethanol (data not shown), this seems not to be caused by inactivation of these proteins.

A phenylalanine (F) is tolerated at every position without any noticeable effect on the import efficiency (Table I). It might be that this amino acid is functional at any position as long as the other two positions are still conserved according to the "optimal" sequence SKL. This "two out of three ain't bad" rule predicted that if we mutated the entire tripeptide to phenylalanine residues, the import efficiency would decrease. This was indeed the case, although the NH-MDH3-FFF protein was still imported to some extent and able to complement the Δ*mdh3* mutant.

We also tested the tripeptide ANL, which is the putative (nonconsensus) targeting signal of human peroxisomal catalase. We observed rather efficient import of MDH3-ANL, suggesting that this tripeptide functions as a valid PTS1 signal.

Dimerization of MDH3—Recently, it has been shown by several elegant studies that proteins can be imported into peroxisomes in a folded (multimeric) state (13–15, 31). For example, thiolase without a peroxisomal targeting signal (thiolaseΔPTS2) can be imported when expressed in wild-type cells but not when it is expressed in a thiolase disruption strain (13). This strongly suggested that thiolaseΔPTS2 is imported as a dimer with wild-type thiolase ("piggy-back") and hence that complete unfolding is probably not required for import into peroxisomes. A similar experiment was reported in Ref. 14. Using the same experimental setup, we tested whether dimerization of MDH3 also precedes import. To that end we transformed the plasmid pNH-MDH3ΔPTS1 to wild-type cells. The NH-MDH3ΔPTS1 protein not only contains an epitope tag to distinguish it from wild-type MDH3 but is also 7 amino acids longer than wild-type MDH3, because the COOH-terminal 5 amino acids were replaced by a peptide of 12 amino acids (Fig. 1). Because this larger protein can easily be distinguished from the shorter wild-type protein, it enabled us to verify that no recombination events had occurred between the pNH-

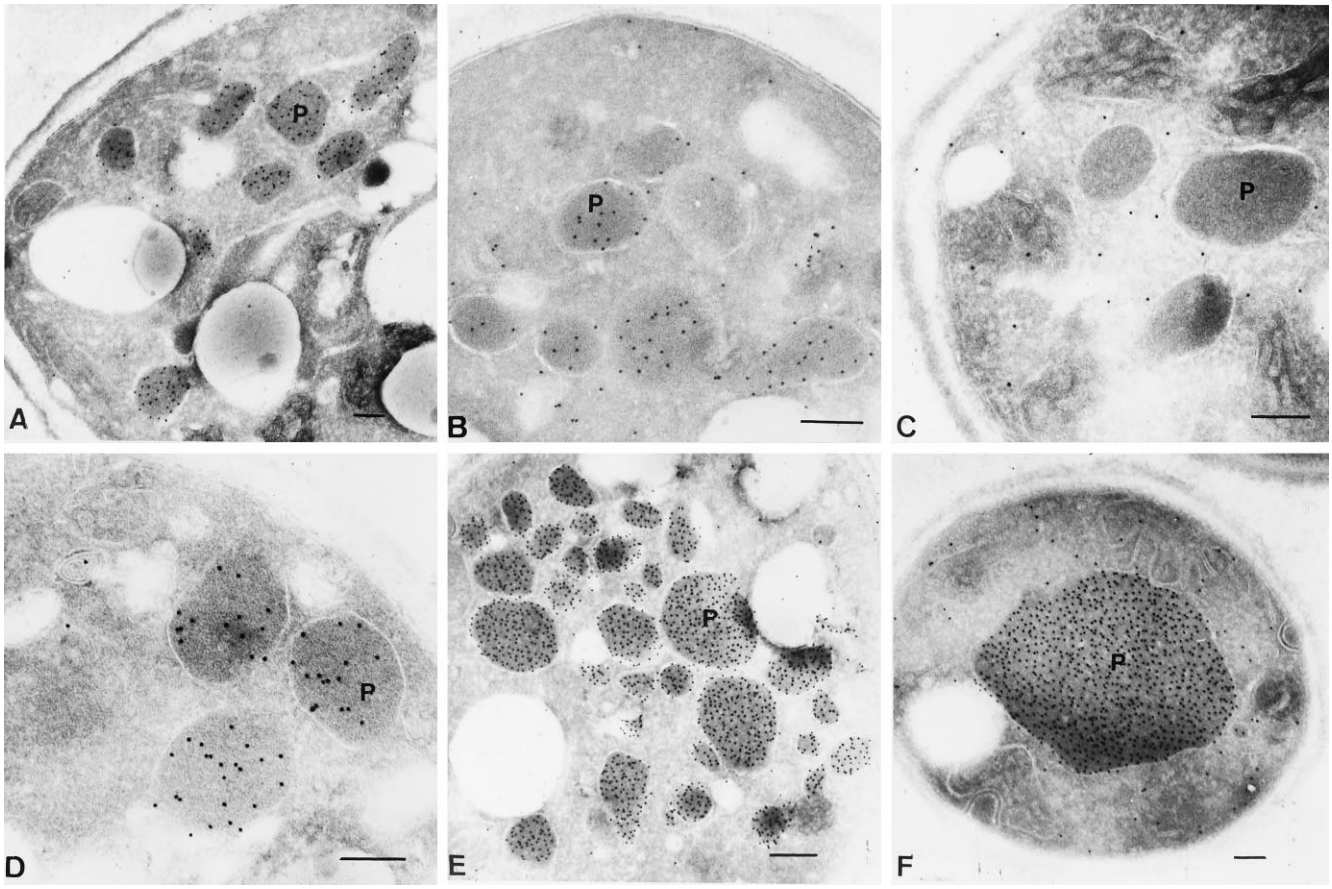

FIG. 4. **Electronmicroscopy analysis of Δ*mdh3* cells expressing NH-MDH3-SKL (*A*), NH-MDH3-SEL (*B*), NH-MDH3ΔKL (*C*), NH-MDH3ΔPTS1/WT-MDH3(2μ) (*D*), and NH-MDH3-SKL(2μ) (*E* and *F*).** Cryosections of cells grown on oleate were labeled using the NH antibody and immunogold particles conjugated with protein A. *P*, peroxisome. *Bars*, 0.2 μm.

MDH3ΔPTS1 plasmid and the endogenous wild-type gene. Such an event could result in an NH-MDH3-SKL gene, which would hamper the analysis. The transformed cells were induced on oleate, and the amount of imported NH-MDH3ΔPTS1 was determined by subcellular fractionation followed by Western blotting using the NH antibody. Surprisingly, in contrast to the earlier reports using different proteins but a similar experimental setup (13, 14), we did not find any NH-MDH3ΔPTS1 in the organellar pellet fraction (Fig. 5). One possibility is that heterodimers were not formed between WT-MDH3 and NH-MDH3ΔPTS1. Therefore, we tested the formation of heterodimers by immunoprecipitations with the NH antibody on lysates of oleate-induced wild-type cells expressing NH-MDH3ΔKL or NH-MDH3ΔPTS1 (Fig. 6). No heterodimer formation between NH-MDH3ΔPTS1 or NH-MDH3ΔKL and WT-MDH3 could be demonstrated (Fig. 6, compare *first* and *third lanes* and *second* and *fourth lanes*). Because it is possible that the modifications at the carboxyl terminus of NH-MDH3ΔPTS1 and NH-MDH3ΔKL inhibited dimerization, we tested the ability of NH-MDH3ΔKL to form dimers by using a sucrose density gradient. We found that this protein had not lost its ability to dimerize (data not shown). In addition, the NH-MDH3ΔKL protein was still active, because it was able to complement the growth defect of a Δ*mdh2* mutant on ethanol (data not shown).

Another explanation for the failure to detect heterodimers is the possibility that heterodimers were disassembled during the import process. Alternatively, Because it has recently been suggested that import of some peroxisomal proteins is much faster than other peroxisomal proteins (32), import of WT-MDH3 might be so efficient that it does not get the chance to dimerize in the cytosol prior to import. We tested these possibilities by repeating the immunoprecipitations on lysates of Δ*pex5* cells transformed with NH-MDH3ΔPTS1 and NH-MDH3ΔKL. Because the PTS1 import is blocked in this mutant, dimerization of MDH3 only takes place in the cytosol. However, we were still unable to detect heterodimers, suggesting that the inability to observe heterodimers was not caused by the import process (data not shown). Taken together, these results hinted at the possibility that dimerization of MDH3 is an efficient process and that dimers are mostly formed between proteins synthesized on the same polyribosome, resulting in the formation of homodimers only. We argued that if this was indeed be the case, we might be able to increase the amount of heterodimers by expressing WT-MDH3 from a multicopy plasmid in cells expressing NH-MDH3ΔPTS1 or NH-MDH3ΔKL. Indeed, we were now able to co-immunoprecipitate WT-MDH3 with the NH antibody in lysates of these double transformants. Up to approximately 30% of NH-MDH3ΔPTS1 or NH-MDH3ΔKL had formed heterodimers with WT-MDH3 (Fig. 6, *lanes 5* and *6*). It can further be seen that recombination of pNH-MDH3ΔPTS1 with pWT-MDH3(2μ) did not noticeably occur, because this would have given an additional band at about the same height as NH-MDH3ΔKL.

To test whether the formed heterodimers could be imported into peroxisomes, wild-type cells transformed with pNH-MDH3ΔPTS1/pWT-MDH3(2μ) were grown on oleate and fractionated. The organellar and cytosolic fractions were used for Western blotting using the NH antibody. At least 30% of NH-MDH3ΔPTS1 was now present in the organellar pellet fraction, which is in good agreement with the observed amount of heterodimers formed. To exclude that NH-MDH3ΔPTS1 was not trapped in pelletable protein aggregates caused by the overex-

TABLE I
Targeting of MDH3-PTS1 variants

Analysis of the targeting efficiency of the various MDH3-PTS1 variants is shown. Cells were grown on oleate, fractionated, and the organellar pellet and cytosolic supernatant fractions were used for Western blotting with the NH antibody. After incubation with ^{125}I-labeled protein A, import was quantified with a PhosphorImager. Import efficiency: ++, more than 80%; +, between 80 and 50%; −, between 20 and 5%; − −, less than 5% import.

XKL	Import	SXL	Import	SKX	Import	XXX	Import
EKL	++	SAL	+	SKD	−−	ΔKL	−−
FKL	++	SEL	+	SKE	−	FFF	−
GKL	++	SFL	++	SKF	++	ANL	+
IKL	++	SHL	++	SKL	++		
KKL	++	SKL	++	SKN	−		
LKL	++	SRL	++	SKP	−		
MKL	++	SSL	+	SKR	−−		
NKL	++	SYL	++	SKS	−		
PKL	++			SKT	−		
SKL	++			SKV	++		
TKL	++						

pression of WT-MDH3, we used the same cells for immunoelectronmicroscopy. This confirmed that NH-MDH3ΔPTS1 was indeed imported into peroxisomes when these cells were cotransformed with pWT-MDH3(2μ) (Fig. 4*D*). Surprisingly, the overexpression of WT-MDH3 did not lead to aggregation of the protein in the cytosol. The import machinery appeared to be perfectly capable of dealing with this large amount of PTS1-containing protein, resulting in the formation of many and very large peroxisomes (Fig. 4*E*). Taken together, the results suggest that folding and dimerization of MDH3 precedes import into the peroxisome.

DISCUSSION

Degeneracy of the PTS1—The carboxyl-terminal peroxisomal targeting signal (PTS1) has been studied by extensive mutational analyses (5–8). In all these cases peroxisomal proteins from other organisms or nonperoxisomal proteins were used as reporters. This resulted in a consensus sequence to which functional PTS1s were expected to conform. Since the formulation of this consensus sequence, more and more peroxisomal proteins were identified whose PTS1-like tripeptide did not fit this consensus sequence. We therefore decided to repeat such an analysis in *S. cerevisiae*, using a peroxisomal protein of *S. cerevisiae* as reporter. Because it has been demonstrated for some PTS1-containing enzymes that deletion of the PTS1 does not block import into peroxisomes (23, 26, 27), we first demonstrated that import of MDH3 is completely dependent upon the presence of its PTS1 and the PTS1 receptor, Pex5p. Furthermore, we showed that this protein could be epitope-tagged without any noticeable effect on its import efficiency. Finally, we demonstrated that varying the COOH terminus of MDH3 did not greatly affect its activity because the nonimported variants were able to complement the growth defect on ethanol of the Δ*mdh2* mutant, which lacks the cytosolic malate dehydrogenase. This has recently also been reported by Ref. 33, which showed that deletion of the SKL tripeptide of MDH3 did not affect its specific activity. Taken together, these findings made MDH3 a reliable model protein for studying the PTS1 requirements.

Although we tested only 29 out of the 60 possible amino acid substitutions, we have taken care to have representative amino acids at all positions by testing at least one positively and one negatively charged amino acid, one hydrophobic and aromatic amino acid, and one polar amino acid at each position. The results we obtained differ in several aspects from previous reports concerning the degeneracy of the PTS1 signal.

(i) We did not find any amino acid substitution at the first position of the tripeptide that affected the import of MDH3. Strictly taken, the targeting signal of MDH3 is therefore not a tripeptide but a dipeptide. Especially the finding that a phenylalanine (F) or a proline (P) is tolerated at this position is surprising, for it is in sharp contrast to the observation that this residue should preferably be small, as has been found for luciferase expressed in mammalian cells (5) or for the chloramphenicol acetyltransferase-glycosomal phosphoglycerate kinase fusion protein expressed in trypanosomes (7). We believe that the observation that many substitutions are tolerated at this position in endogenous peroxisomal proteins is more in line with the reality than the observation that this amino acid should strictly be small. Examples of this greater tolerancy in endogenous proteins are the PTS1 of human alanine:glyoxylate aminotransferase, which is KKL (12), and the PTS1 of peroxisomal fatty acid synthetase of *S. cerevisiae* (FAA2), which is EKL (43). Neither the EKL nor the KKL tripeptide was able to direct luciferase to peroxisomes or glycosomes (5, 8). Similarly, dihydroxyacetone synthase of *Hansenula polymorpha* and rat and feline alanine:glyoxylate aminotransferase are targeted via the tripeptide NKL (34–36). Also this asparagine (N) does not fit the consensus of a small residue at the first position.

(ii) We found no requirement for a basic amino acid at the penultimate position as found for luciferase import in peroxisomes of mammalian cells (5) and as observed to a lesser extent for luciferase import in glycosomes of trypanosomes (8). In agreement with our results are the findings that a positive residue at the penultimate position is also not required for import of the hybrid chloramphenicol acetyltransferase-glycosomal phosphoglycerate kinase protein in glycosomes of trypanosomes (7). It is possible that for *S. cerevisiae* and trypanosomes, the requirements at the penultimate position are more relaxed than for mammalian cells. However, the putative PTS1 of human catalase also does not have a positively charged residue at the second position of its PTS1-like tripeptide (ANL) (37). Again, we therefore propose that in a homologous context many more variants are functional at this position than in a heterologous context. This notion is supported by the observation that human alanine:glyoxylate aminotransferase can be imported into peroxisomes of mammalian cells when the PTS1 of alanine:glyoxylate aminotransferase is replaced by SSL (12). In contrast, luciferase-SSL cannot be imported into peroxisomes of mammalian cells (5). Furthermore, MDH3-SEL is imported into peroxisomes of *S. cerevisiae* with more than 50% efficiency, whereas luciferase-SEL is not imported into peroxisomes of *S. cerevisiae* (20, 28).

(iii) Finally, we found that at the last position only hydrophobic residues result in efficient import. In agreement with previous observations (7, 8), we observed that the ultimate amino acid could also be replaced by an aromatic residue. This result confirms an earlier report that the tripeptide SKF of catalase from *S. cerevisiae* is a legitimate PTS1 signal (26).

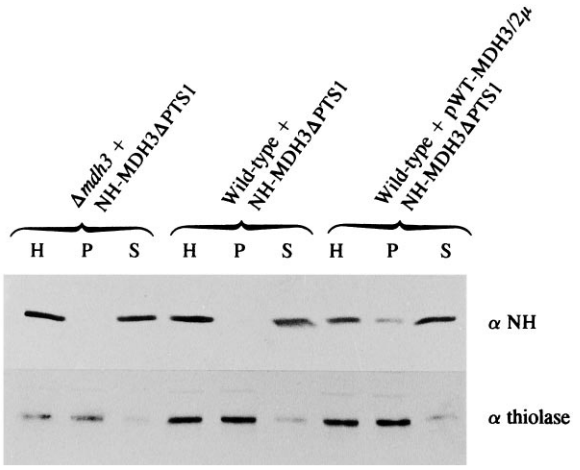

FIG. 5. **Co-import of NH-MDH3ΔPTS1 with WT-MDH3.** The Western blot shows the subcellular distribution of the NH-MDH3ΔPTS1 when expressed in Δ*mdh3* cells, in wild-type cells, and in wild-type cells co-transformed with MDH3-SKL(2μ). The 30,000 × *g* pellet fraction (*P*) represents the organellar fraction, whereas the supernatant (*S*) fraction represents the cytosolic fraction. *H* represents the homogenate before the high speed spin. Comparable volumes were layered in every lane. Proteins were detected using NH antibodies and an alkaline phosphatase-based color staining.

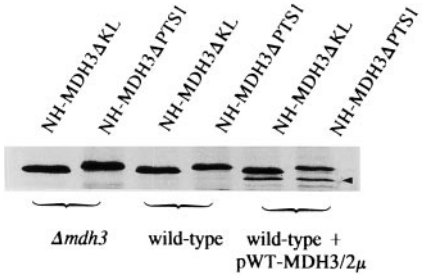

FIG. 6. **SDS-polyacrylamide gel (15%) showing immunoprecipitated NH-MDH3ΔKL and NH-MDH3ΔPTS1 using NH-antibodies.** NH-MDH3ΔKL and NH-MDH3ΔPTS1 were expressed in Δ*mdh3* cells (*first* and *second lanes*), in wild-type cells (*third* and *fourth lanes*), and in wild-type cells co-expressing MDH3-SKL(2μ) (*fifth* and *sixth lanes*). Cells were grown on glycerol, labeled for 2 h, and lysed as described under "Experimental Procedures." Similar results were obtained in oleate-induced cells, except that considerably less labeling was obtained in Δ*mdh3* cells. The *arrowhead* shows the position of WT-MDH3.

Importance of the Homologous Context—It is conceivable that the originally defined consensus PTS1 (a small amino acid at the first position, a positively charged amino acid at the second position, and a hydrophobic amino acid at the third position) as delineated for a heterologously expressed protein (5) represents the most favorable PTS1 signal. This is supported by the observation that most PTS1 signals found in nature are consistent with this consensus sequence. In addition, we showed in this study that a phenylalanine (F) was tolerated at every position in the PTS1 of MDH3, without affecting the import efficiency, whereas a PTS1 consisting of the tripeptide FFF was hardly able to direct MDH3 to peroxisomes. This illustrates that only a limited number of mutations within the ideal SKL tripeptide is tolerated.

It is quite often observed that certain defined targeting signals work better with one reporter protein than another. This can be explained by assuming that a targeting signal is better exposed in certain reporter proteins than others. Our results for the PTS1 seem to stress the importance of the proper reporter even further, because they imply that a much broader range of PTS1 variants is functional in endogenous peroxisomal proteins compared with heterologously expressed peroxisomal proteins. This explains many of the encountered failures to demonstrate that certain PTS1-resembling tripeptides are able to direct a reporter protein to peroxisomes. This inability has often been explained by proposing either protein context dependence or species divergence. The importance of the homologous context explains both the observed species divergence, as well as the observed context dependence. This is probably best illustrated by the following example. The SSL tripeptide of glycosomal phosphoglycerate kinase (38) was not able to direct a reporter protein (chloramphenicol acetyltransferase) to peroxisomes of mammalian cells, whereas chloramphenicol acetyltransferase-SKL was imported into these peroxisomes (5, 7). Because both chloramphenicol acetyltransferase variants were imported into glycosomes of trypanosomes, this strongly suggests species divergence in the ability to recognize the SSL tripeptide. However, Motley *et al.* (12) recently demonstrated that peroxisomes of mammalian cells were perfectly capable of importing the alanine:glyoxylate aminotransferase-SSL protein. Because luciferase-SSL was not imported in these peroxisomes (5), this observation would suggest that the ability to recognize the SSL tripeptide is dependent on the protein context. Similarly, glycosomal phosphoglycerate kinase has been expressed in *S. cerevisiae* but failed to be imported into peroxisomes (39). Our results in the present study show, however, that SSL can be recognized by the peroxisomal import system of *S. cerevisiae* provided that it is attached to an endogenous protein. The observations that signals behave either in a species-dependent manner or in a context-dependent manner or even in both are consistent with the notion that the homologous context is very important.

What could be the rationale of the importance of the homologous context? It is conceivable that the interaction of peroxisomal matrix proteins with components of the peroxisomal import machinery, particularly Pex5p, is not restricted to the PTS1 but that interactions with other parts of the PTS1-containing protein are also required to ascertain that a PTS1 variant is indeed recognized as such. These "accessory sequences" may directly precede the PTS1, because it has been shown several times that a certain PTS1 tripeptide was not sufficient to direct a reporter protein to peroxisomes, whereas a hybrid protein with a longer COOH-terminal fusion was able to do so (7, 26, 28). The accessory sequences may also be further upstream of the PTS1. The observations that folding and dimerization of both thiolase (13) and MDH3 (this study) already take place in the cytoplasm suggest that this is not restricted to some exceptional cases but rather is the default pathway for many (oligomeric) peroxisomal proteins. Therefore, it is conceivable that accessory sequences are formed by conformational epitopes within the folded protein. The interaction of these epitopes with the PTS1 receptor or with other components of the protein import machinery may be of decisive importance to determine whether a PTS1 variant is functional or not. Such interactions may be lacking when heterologously expressed proteins are used, when nonperoxisomal proteins are used, or even when mutations upstream of the PTS1 are introduced in a protein. We assume that during evolution the "ideal" PTS1 sequences may have become more degenerated, depending on the contribution of accessory sequences in binding the receptor. If this contribution is only minimal, a strong selective pressure is put on the conservation of an optimal PTS1. However, when the contribution of the accessory sequences is large, the selective pressure on keeping an optimal PTS1 is lost. There are some indications that the interactions mediated by accessory sequences are so strong that they can function as a true (though not very efficient) targeting signal. For instance, it has been reported that the PTS1-containing proteins catalase

and carnitine acetyltransferase of *S. cerevisiae* are still imported into peroxisomes when their PTS1 signal has been deleted. However, deletion of the PTS1 receptor blocks their import completely (17, 23, 26). This may indicate that these proteins contain additional domains that interact with the PTS1 receptor. Indeed, an interaction between carnitine acetyltransferase lacking its PTS1 and the PTS1 receptor Pex5p could be demonstrated using the two-hybrid assay (23). We were unable to demonstrate the existence of additional Pex5p interacting sequences in MDH3, because the two-hybrid interaction between MDH3-SKL and Pex5p was rather weak and already undetectable when the SKL tripeptide was replaced by SEL (40). Support for the existence of accessory sequences involved in binding the PTS1 receptor also comes from the observation that many nonperoxisomal proteins have carboxyl termini that fit with our observed relaxed "consensus" for functional PTS1 (di)peptides. Therefore, additional criteria are required to distinguish between genuine peroxisomal proteins and nonperoxisomal proteins.

The existence of accessory sequences that mediate the binding of the PTS1 parallels the findings for nuclear protein import. Here, it has also been observed that flanking sequences of the nuclear localization sequence (NLS) modulate the import of a protein and, like the PTS1, the NLS does not have a strict consensus sequence (reviewed in Ref. 41). Moreover, both organelles can import folded proteins, and because we found that Pex5p is predominantly cytoplasmic (19), both import systems make use of mobile receptors.

Our analysis of the PTS1 of MDH3 demonstrates that many PTS1-like signals are functional in a homologous context. Consequently, the definition of a targeting signal that it should be (i) required for import and (ii) sufficient to direct an otherwise cytosolic protein to peroxisomes may fall short for the PTS1 signal. Therefore, whether import of a peroxisomal protein truly relies on a PTS1 like signal is best tested by determining whether import requires the presence of the PTS1 receptor Pex5p. Our documentation of functional PTS1 motifs in a endogenous protein of *S. cerevisiae* may furthermore be useful to select putative peroxisomal proteins from the Yeast Genome Sequencing data base. Many of such proteins may previously have been overlooked, because their carboxyl termini did not fit the originally defined consensus of the PTS1.

Acknowledgments—We are grateful to B. Metzig, L. Kwast, E. Hettema, A. Motley, and I. Braakman for technical support and/or stimulating discussions. We also thank L. McAlister-Henn for providing us with the Δ*mdh2* strain.

Addendum—After this manuscript was submitted, a new nomenclature was adopted for the *PAS* genes (42). Proteins involved in peroxisome biogenesis are now designated as peroxins. The new names for *ScPAS10* and *ScPAS7* are now *ScPEX5* and *ScPEX7*, respectively.

REFERENCES

1. Gould, S. J., Keller, G.-A., and Subramani, S. (1987) *J. Cell Biol.* **105,** 2923–2931
2. Keller, G. A., Gould, S. J., Deluca, M., and Subramani, S. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3264–3268
3. Osumi, T., Tsukamoto, T., Hata, S., Yokota, S., Miura, S., Fujiki, Y., Hijikata, M., Miyazawa, S., and Hashimoto, T. (1991) *Biochem. Bioph. Res. Commun.* **181,** 947–954
4. Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. (1991) *EMBO J.* **10,** 3255–3262
5. Gould, S. J., Keller, G.-A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) *J. Cell Biol.* **108,** 1657–1664
6. Swinkels, B. W., Gould, S. J., and Subramani, S. (1992) *FEBS Lett.* **305,** 133–136
7. Blattner, J., Swinkels, B., Dorsam, H., Prospero, T., Subramani, S., and Clayton, C. (1992) *J. Cell Biol.* **119,** 1129–1136
8. Sommer, J. M., Cheng, Q. L., Keller, G. A., and Wang, C. C. (1992) *Mol. Biol. Cell* **3,** 749–759
9. De Hoop, M. J., and AB, G. (1992) *Biochem. J.* **286,** 657–669
10. Roggenkamp, R. (1992) *Cell Biochem. Funct.* **10,** 193–199
11. Subramani, S. (1993) *Annu. Rev. Cell Biol.* **9,** 445–478
12. Motley, A., Lumb, M. J., Oatey, P. B., Jennings, P. R., De Zoysa, P. A., Wanders, R. J. A., Tabak, H. F., and Danpure, C. J. (1995) *J. Cell Biol.* **131,** 95–109
13. Glover, J. R., Andrews, D. W., and Rachubinski, R. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10541–10545
14. McNew, J. A., and Goodman, J. M. (1994) *J. Cell Biol.* **127,** 1245–1257
15. Walton, P. A., Hill, P. E., and Subramani, S. (1995) *Mol. Biol. Cell* **6,** 675–683
16. Van Roermund, C. W. T., Elgersma, Y., Singh, N., Wanders, R. J. A., and Tabak, H. F. (1995) *EMBO J.* **14,** 3480–3486
17. Van der Leij, I., Franse, M. M., Elgersma, Y., Distel, B., and Tabak, H. F. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11782–11786
18. Van der Leij, I., Van den Berg, M., Boot, R., Franse, M., Distel, B., and Tabak, H. F. (1992) *J. Cell Biol.* **119,** 153–162
19. Elgersma, Y., Kwast, L., Klein, A., Voorn-Brouwer, T., Van den Berg, M., Metzig, B., America, T., Tabak, H. F., and Distel, B. (1996) *J. Cell Biol.*, in press
20. Elgersma, Y., Van den Berg, M., Tabak, H. F., and Distel, B. (1993) *Genetics* **135,** 731–740
21. Gietz, R. D., and Sugino, A. (1988) *Gene* (*Amst.*) **74,** 527–534
22. Gould, S. J., Keller, G. A., Schneider, M., Howell, S. H., Garrard, L. J., Goodman, J. M., Distel, B., Tabak, H. F., and Subramani, S. (1990) *EMBO J.* **9,** 85–90
23. Elgersma, Y., Van Roermund, C. W. T., Wanders, R. J. A., and Tabak, H. F. (1995) *EMBO J.* **14,** 3472–3479
24. Van Roermund, C. W. T., Van den Berg, M., and Wanders, R. J. A. (1995) *Biochim. Biophys. Acta* **1245,** 348–358
25. Steffan, J. S., and McAlister-Henn, L. (1992) *J. Biol. Chem.* **267,** 24708–24715
26. Kragler, F., Langeder, A., Raupachova, J., Binder, M., and Hartig, A. (1993) *J. Cell Biol.* **120,** 665–673
27. Sommer, J. M., Nguyen, T. T., and Wang, C. C. (1994) *FEBS Lett.* **350,** 125–129
28. Distel, B., Gould, S. J., Voorn-Brouwer, T., Van den Berg, M., and Tabak, H. F. (1992) *New Biologist* **4,** 157–165
29. Marzioch, M., Erdmann, R., Veenhuis, M., and Kunau, W. H. (1994) *EMBO J.* **13,** 4908–4918
30. Zhang, J. W., and Lazarow, P. B. (1995) *J. Cell Biol.* **129,** 65–80
31. Hausler, T., Stierhof, Y. D., Wirtz, E., and Clayton, C. (1996) *J. Cell Biol.* **132,** 311–324
32. Szilard, R. K., Titorenko, V. I., Veenhuis, M., and Rachubinski, R. A. (1995) *J. Cell Biol.* **131,** 1453–1469
33. McAlister-Henn, L., Steffan, J. S., Minard, K. I., and Anderson, S. L. (1995) *J Biol Chem.* **270,** 21220–21225
34. Hansen, H., Didion, T., Thiemann, A., Veenhuis, M., and Roggenkamp, R. (1992) *Mol. & Gen. Genet.* **235,** 269–278
35. Oda, T., Funai, T., and Ichiyama, A. (1990) *J. Biol. Chem.* **265,** 7513–7519
36. Lumb, M. J., Purdue, P. E., and Danpure, C. J. (1994) *Eur. J. Biochem.* **221,** 53–62
37. Quan, F., Korneluk, R. G., Tropak, M. B., and Gravel, R. A. (1986) *Nucleic Acids Res.* **14,** 5321–5333
38. Osinga, K. A., Swinkels, B. W., Gibson, W. C., Borst, P., Veeneman, G. H., Van Boom, J. H., Michels, P. A. M., and Opperdoes, F. R. (1985) *EMBO J.* **4,** 3811–3817
39. Swinkels, B. (1989) *Biogenesis of Glycosomes (Microbodies) in Trypanosomes*. Ph.D. Thesis, University of Amsterdam, The Netherlands
40. Tabak, H. F., Elgersma, Y., Hettema, E., Franse, M. M., Voorn-Brouwer, T., and Distel, B. (1995) *Cold Spring Harbor Symp. Quant. Biol.* **60,** 649–655
41. Silver, P. A. (1991) *Cell* **64,** 489–497
42. Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J. A. K. W., Kunau, W.-H., Lazarow, P. B., Mannaerts, G. P., Moser, H., Osumi, T., Rachubinski, R. A., Roscher, A., Subramani, S., Tabak, H. F., Valle, D., Van der Klei, I., Van Veldhoven, P. P., and Veenhuis, M. (1996) *J. Cell Biol.*, in press
43. Hettema, E. H., Van Roermund, C. W. T., Distel, B., Van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R. J. A., and Tabak, H. F. (1996) *EMBO J.* **15,** 3813–3822

Analysis of the Carboxyl-terminal Peroxisomal Targeting Signal 1 in a Homologous Context in *Saccharomyces cerevisiae*

Ype Elgersma, Arnold Vos, Marlene van den Berg, Carlo W. T. van Roermund, Peter van der Sluijs, Ben Distel and Henk F. Tabak

J. Biol. Chem. 1996, 271:26375-26382.
doi: 10.1074/jbc.271.42.26375