

Mutational analysis of the N-terminal topogenic signal of watermelon glyoxysomal malate dehydrogenase using the heterologous host *Hansenula polymorpha*

[microbodies (glyoxysomes, peroxisomes)/mitochondria/*Citrullus vulgaris*/isoenzymes/protein targeting]

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ABSTRACT We have studied the significance of the N-terminal presequence of watermelon (*Citrullus vulgaris*) glyoxysomal malate dehydrogenase [gMDH; (S)-malate:NAD⁺ oxidoreductase; EC 1.1.1.37] in microbody targeting. The yeast *Hansenula polymorpha* was used as heterologous host for the *in vivo* expression of various genetically altered watermelon MDH genes, whose protein products were localized by immunocytochemical techniques. It is shown that the presequence of gMDH is essential and sufficient for peroxisomal targeting; it can target the mature part of the mitochondrial MDH to microbodies, whereas deletion of the presequence results in accumulation of the mature form of gMDH in the cytosol. Alignment of the N termini of several peroxisomal proteins that are assumed to contain a peroxisomal targeting signal at the N terminus (PTS2) suggested the consensus sequence RL-X₅-HL. A similar motif is present in the presequence of watermelon gMDH—namely, ¹⁶RI-X₅-¹⁷HL. Mutational analysis revealed that substitutions of ¹⁶RI into DD or ¹⁷HL into DE destroyed the topogenic information, whereas substitutions of ²⁵M into I and ²⁶EE into LV did not. By combining our data with recent analyses of others on the presequences of mammalian thiolases, it is concluded that the peroxisomal targeting information of PTS2 is contained in the consensus sequence RL/I-X₅-HL. In contrast to the higher plant and mammals, the *Hansenula* yeast peroxisomes seem to lack an enzyme capable of removing microbody presequences of higher eukaryotes.

In plant tissues several forms of malate dehydrogenase [MDH; (S)-malate:NAD⁺ oxidoreductase; EC 1.1.1.37] exist whose specific physiological functions differ with their subcellular locations. At present three cytosolic MDH isoforms are discriminated that function in the malate shuttle and gluconeogenesis. In addition, one MDH is located in the mitochondrial matrix as a component of the tricarboxylic acid cycle and one is in microbodies (glyoxysomes, peroxisomes) where it functions in the glyoxylate cycle or the photorespiratory pathway (1). To ensure that the various metabolic pathways can function in the plant cell, distinct mechanisms exist that control the targeting of the different isoforms of MDH to their correct subcellular destination. Both organelle-bound MDHs are synthesized with an N-terminal presequence, comprising 37 amino acids for the glyoxysomal precursor form (pre-gMDH) (2) and 27 amino acids for the mitochondrial one (pre-mMDH) (3); both presequences are cleaved off upon import (1). The mitochondrial presequence can form a positively charged amphiphilic α -helix, typical for presequences of mitochondrial matrix precursors (4). In the case of microbodies (peroxisomes, glyoxysomes), two dif-

ferent topogenic signals have been identified so far that are necessary and sufficient to direct cytosolic precursors of matrix enzymes to their target organelle. One of these, designated PTS1, includes the C-terminal tripeptide SKL (5, 6) and is highly conserved throughout the eukaryotic kingdom (7, 8). Subsequently, evidence was obtained that in a few matrix proteins (e.g., 3-ketoacyl-CoA-thiolases of higher eukaryotes) the topogenic information (now designated PTS2) is located in the N terminus of the protein (9). Similarly, the N-terminal presequence of watermelon gMDH is assumed to contain the targeting information (2). Alignment of these proteins with several other microbody matrix proteins that do not contain a PTS1 (9) suggested a consensus sequence, RL-X₅-HL, at the N terminus; a comparable motif is present in the presequence of watermelon gMDH (2, 9).

Recently, we have introduced the gene encoding the watermelon (*Citrullus vulgaris*) pre-gMDH into the methylotrophic yeast *Hansenula polymorpha*. We demonstrated that the pre-gMDH protein synthesized in the heterologous host is normally sorted to peroxisomes (10). This has opened the way to analyze the targeting information of this plant microbody enzyme in depth. For this purpose we studied in *H. polymorpha* the fate of the protein products of different genetically modified MDH genes, which, among others, code for various site-directed mutations in the presequence. The results of these studies are presented.

MATERIALS AND METHODS

Organisms and Growth Conditions. Untransformed *H. polymorpha* A16 (11) and transformants carrying the various MDH genes under control of the *H. polymorpha* alcohol oxidase promoter (P_{MOX}) on the pHIPX4 vector were grown in batch cultures on mineral medium containing methanol (0.5%) as carbon source in the presence of ammonium sulfate (0.2%) as nitrogen source (12).

Construction of cDNA Clones Encoding Hybrid and Mutated MDH Proteins. Two fusion genes (pre-g::mMDH and pre-m::gMDH) and site-directed mutations in the glyoxysomal presequence (¹⁶RI→DD; ¹⁷HL→DE; ²⁵M→I and ²⁶EE→LV) were generated using the “gene splicing by overlap extension” method (13, 14). Sequences coding for watermelon pre-gMDH (2) and pre-mMDH (3) were inserted as 1.3-kb *Not* I–*Sal* I fragments in pGEM-5Zf(+) (Promega) and were used as template in PCR experiments. For construction of the gene fusions and the introduction of mutations in the pre-

Abbreviations: MDH, malate dehydrogenase; gMDH, glyoxysomal MDH; mMDH, mitochondrial MDH.

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quence both sense and antisense primers were designed, containing the modified sequence. Two PCR amplification experiments were performed using these primers in combination with the reverse sequencing primer and the universal sequencing primer, respectively. In a third amplification step the hybrid/mutated gene was produced by mixing the products of the first two reactions and adding an excess of universal and reverse primers. In addition, a gene coding for mature gMDH was constructed. A 126-bp *Not* I–*Sac* I fragment from pre-gMDH (coding for the transit peptide and the first 5 amino acids of mature gMDH) was replaced by a 25-bp double-stranded synthetic DNA fragment. This fragment codes for the first 5 amino acids of mature gMDH preceded by an ATG initiation codon and contains a *Not* I (5') and a *Sac* I (3') compatible overhang. All mutant/hybrid genes were checked by sequence analysis and, including the two wild-type genes (pre-gMDH and pre-mMDH), subcloned as 1.3-kb *Not* I (sticky ends filled in)–*Sal* I fragments in *Hind*III (sticky ends filled in)–*Sal* I-digested pHIPX4 (Fig. 1), placing the genes under control of the *H. polymorpha* P_{MOX}.

Construction of the *H. polymorpha* Expression Vector pHIPX4. pHIPX4 was constructed using the following DNA fragments (numbers indicate nucleotide positions on pHIPX4) [(*) indicates sticky ends filled in using Klenow enzyme]: 1–42, *Hind*III–*Sma* I fragment from pTZ19R containing part of the polylinker (15); 43–441, *Sma* I–*Hind*III(*) fragment containing the *H. polymorpha* amine oxidase terminator region (T_{AMO}) (16); 442–820, *Bgl* II(*)–*Hind*III(*) fragment from pMA91 containing the *Saccharomyces cerevisiae* phosphoglycerate kinase terminator region (T_{PGK}) (17); 821–3041, *Hind*III(*)–*Bgl* II fragment containing the *LEU2* gene from *S. cerevisiae* from YEp13 (18). These sites originate from ligation of the original *Xho* I(*) and *Sal* I(*) sites in YEp13 to *Hind*III(*) and *Bgl* II(*) sites, respectively: 3042–3508, *Bgl* II–*Nru* I *H. polymorpha* genomic DNA fragment containing the terminator region of the alcohol oxidase gene (19); 3509–5565, *Stu* I–*Bam*HI(*) fragment from pOK12 (20); 5566–7072, *Sal* I(*)–*Hind*III fragment containing the *H. polymorpha* P_{MOX} (19). The *Hind*III site was created by site-directed mutagenesis at the translation initiation site of the alcohol oxidase gene using the following oligonucleotide (mutated bases in uppercase letters, *Hind*III site underlined): 5'-ggaatggcAaGCttgtttttgtac-3'.

Recombinant DNA Manipulations. All recombinant DNA manipulations were performed using standard methods (21). *Escherichia coli* DH5 α , used for plasmid amplification, was transformed by the Ca²⁺ method. *H. polymorpha* A16 was transformed by electroporation (22).

Biochemical Methods. Preparation of crude extracts and determination of protein concentrations and specific activity

of MDH activity were carried out as described (10). Crude extracts were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), followed by Western blotting using polyclonal antibodies generated against watermelon gMDH and mMDH.

Electron Microscopy. Immunocytochemistry was performed on ultrathin sections of Unicryl-embedded cells (23) using polyclonal antibodies raised against watermelon gMDH and mMDH and goat anti-rabbit antibodies conjugated with gold (Amersham) according to instructions of the manufacturer.

RESULTS

MDH Expression. All transformants of *H. polymorpha* containing the different MDH genes (pre-gMDH, pre-mMDH, pre-g::mMDH, pre-m::gMDH, gMDH, and the mutated pre-gMDH genes ¹⁰RI→DD, ¹⁷HL→DE, ²⁵M→I, and ²⁶EE→LV) grew well on glucose and methanol at rates comparable to those of the untransformed parental strain. For expression of the various watermelon MDH genes, transformants were grown on methanol to obtain full derepression of the P_{MOX}. In crude extracts of these cells the total MDH activity had significantly increased compared to that of control cells, indicating that watermelon MDH is synthesized and enzymatically active in the transformants (Table 1). Only one exception was encountered—namely, the strain transformed with pre-g::mMDH, which showed a specific activity similar to that of control levels. Apparently, correct folding and activation of the protein are prevented inside *H. polymorpha* peroxisomes, probably as a result of the fact that removal of the presequence did not take place (detailed below).

Further proof for the synthesis of watermelon MDH in *H. polymorpha* was obtained by Western blot analysis using polyclonal antisera raised against watermelon gMDH (α -gMDH; Fig. 2) and the mitochondrial isoenzyme (α -mMDH; Fig. 3). As reported earlier (10), the antibodies raised against the watermelon mMDH recognize the 35-kDa mMDH of *H. polymorpha* and a 41-kDa cytosolic protein (Fig. 3, lane 1), whereas the antibodies against watermelon gMDH (α -gMDH) do not recognize any protein in crude extracts of untransformed *H. polymorpha* (Fig. 2, lane 1). In crude extracts of *H. polymorpha* transformed with wild-type watermelon pre-gMDH or pre-gMDH, mutated in the presequence, a protein band with a molecular mass of 41 kDa is apparent, which represents the unprocessed form of pre-gMDH (Fig. 2, lanes 2, 6–9, and 11).

Table 1. Total MDH specific activities in crude extracts prepared from the various *H. polymorpha* transformants grown on methanol

| Transformant | Enzyme activity, units/mg of protein |
|---|--------------------------------------|
| Parental strain [A16] | 23.0 \pm 1.0 |
| Control [pre-gMDH (²⁵ M→I), grown on glucose] | 25.0 \pm 1.1 |
| Pre-gMDH | 37.6 \pm 0.8 |
| Pre-mMDH | 56.7 \pm 3.3 |
| Pre-g::mMDH | 23.3 \pm 0.1 |
| Pre-m::gMDH | 30.7 \pm 2.3 |
| Pre-gMDH (¹⁷ HL→DE) | 70.0 \pm 2.5 |
| Pre-gMDH (²⁶ EE→LV) | 42.4 \pm 3.0 |
| Pre-gMDH (²⁵ M→I) | 34.6 \pm 2.0 |
| Pre-gMDH (¹⁰ RI→DD) | 50.0 \pm 2.1 |
| gMDH | 42.6 \pm 1.1 |

Enzyme activities are expressed as mean \pm SEM from duplicate determinations.

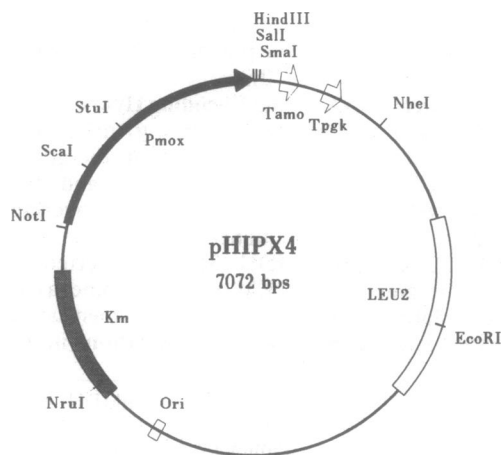


FIG. 1. Physical map of plasmid pHIPX4.

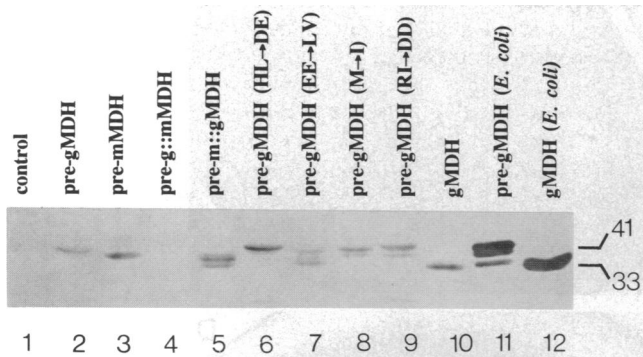


FIG. 2. Western blot of crude extracts of transformed cells of *H. polymorpha* synthesizing watermelon MDH (lanes 2–10) and of extracts prepared from *E. coli* overexpressing either pre-gMDH (lane 11) or mature gMDH (lane 12). As a control (lane 1), a transformed strain [pre-gMDH ($^{25}\text{M}\rightarrow\text{I}$)] grown on glucose was used. On each lane 40 μg of protein was loaded. The blot was decorated with α -gMDH antibodies. Molecular mass is indicated in kDa.

Pre-mMDH is recovered in a protein band with an apparent molecular mass of 38 kDa, suggesting that the mitochondrial presequence is processed in *H. polymorpha* (Fig. 2, lane 3; Fig. 3, lane 2). Although cross-reaction of α -gMDH antibodies with watermelon mMDH is relatively weak (10), mMDH was readily detected due to the high expression level of pre-mMDH in *H. polymorpha*. In crude extracts of cells synthesizing pre-g::mMDH a protein band of ≈ 42 kDa was recognized by α -mMDH antibodies. This indicates that the glyoxysomal presequence is not cleaved when fused to the mature part of mMDH (Fig. 3, lane 3). Pre-m::gMDH is recovered as a major protein band of 33 kDa (Fig. 2, lane 5). The mature gMDH without presequence is synthesized with the expected apparent molecular mass of 33 kDa (Fig. 2, lane 10 vs. lane 12). Pre-gMDH (predominant band of 41 kDa) and mature gMDH (33 kDa), overexpressed in *E. coli*, are shown for size comparison (Fig. 2, lanes 11 and 12).

In summary, all watermelon MDHs are expressed in *H. polymorpha*. Upon comparison of the apparent molecular masses of the various MDH proteins synthesized, it can be concluded that the mitochondrial presequence is processed in *H. polymorpha* irrespective of whether the mature polypeptide chain of the mMDH or gMDH is fused to the mitochondrial presequence. In contrast, none of the proteins containing the presequence of gMDH is cleaved in *H. polymorpha*.

MDH Location Studies. The subcellular location of the various MDH proteins was determined by immunocytochemistry. Immunogold experiments were performed (Fig. 4) on thin sections of methanol/ammonium sulfate-grown cells

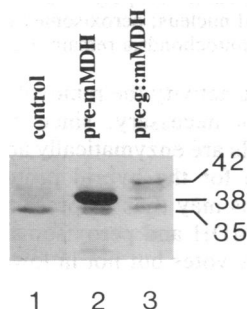


FIG. 3. Western blot of crude extracts of transformed *H. polymorpha* synthesizing watermelon MDH (lanes 2 and 3). As a control (lane 1), a transformed strain [pre-gMDH ($^{25}\text{M}\rightarrow\text{I}$)] grown on glucose was used. On each lane 40 μg of protein was loaded. The blot was decorated with α -mMDH antibodies. Molecular mass is indicated in kDa.

using specific antiserum against gMDH (α -gMDH) or mMDH (α -mMDH) and goat anti-rabbit antibodies conjugated with gold. Control experiments indicated that α -gMDH antisera did not recognize any homologous *H. polymorpha* protein under the experimental conditions used (Fig. 4A). Neither did α -mMDH antiserum recognize any homologous *H. polymorpha* protein in thin sections. As expected, pre-gMDH sorted to peroxisomes of *H. polymorpha* (10) (Fig. 4B). On the other hand, watermelon mMDH was correctly targeted to mitochondria of *H. polymorpha* (Fig. 4C). That both the glyoxysomal and mitochondrial topogenic information functions normally in *H. polymorpha* was also indicated from the results of experiments in which both signals were interchanged: pre-g::mMDH was exclusively targeted to peroxisomes, whereas pre-m::gMDH was solely observed in mitochondria (Fig. 4D and E). When the gene encoding the mature gMDH was expressed in *H. polymorpha*, the resulting protein was found in the cytosol (Fig. 4H), demonstrating that the presequence was indeed essential for import into peroxisomes. To further elucidate the specific topogenic information, present in this presequence, different directed mutations were made and analyzed for their effect on peroxisomal targeting. Both mutations in the nonconserved region—namely, replacement of $^{25}\text{M}\rightarrow\text{I}$ or $^{26}\text{EE}\rightarrow\text{LV}$ —did not affect peroxisomal targeting; in both cases the mutated pre-gMDH protein was present inside peroxisomes (Fig. 4F; $^{25}\text{M}\rightarrow\text{I}$, not shown). However, alterations in the conserved motif of the presequence destroyed the topogenic information; this was indicated by the finding that the protein products of each of the two mutated gMDH genes, $^{17}\text{HL}\rightarrow\text{DE}$ (Fig. 4G) and $^{10}\text{RI}\rightarrow\text{DD}$ (not shown), in methanol-grown *H. polymorpha* were found in the cytosol.

DISCUSSION

This paper describes the *in vivo* analysis of the targeting signal of watermelon gMDH using the methylotrophic yeast *H. polymorpha* as heterologous host. In general, microbody matrix enzymes are synthesized at their final size and have their topogenic information contained in the mature protein. In fact, gMDH in germinating fat-storing seedlings together with different mammalian peroxisomal 3-ketoacyl-CoA thiolases are the only microbody proteins known so far that are synthesized as higher molecular mass precursors. The above proteins do not contain an SKL-like targeting signal (PTS1) at the mature C terminus but, instead, display the presumed PTS2 consensus motif in the presequence (2, 9). We could demonstrate that the watermelon glyoxysomal presequence is sufficient and essential for targeting, because it directs the mMDH as a passenger protein (pre-g::mMDH) to the microbodies, whereas mature gMDH, lacking the presequence, remains in the cytosol. The mature part of the glyoxysomal precursor protein apparently does not contain topogenic information, because the mitochondrial presequence did target the mature gMDH polypeptide chain to the mitochondria (pre-m::gMDH).

Mutations in either ^{10}RI (which is similar to RL) or ^{17}HL destroyed the targeting information: as a result, these mutated precursor proteins were mislocalized into the cytosol. Mutations in the $^{26}\text{EESS}$ motif in the gMDH presequence, however, which could represent a targeting motif deduced from its similarity with the peroxisomal thiolase presequence (2), did not influence peroxisomal import. These data are consistent with the findings for rat thiolase, which showed that truncated versions of the presequence, missing either part or all of the PTS2 consensus sequence, failed to target a reporter protein into microbodies (24, 25). An exchange of the conserved histidine in the presequence rat peroxisomal thiolase was sufficient to abolish the import; of 9 amino acids tested, only glutamine could partially substitute for the

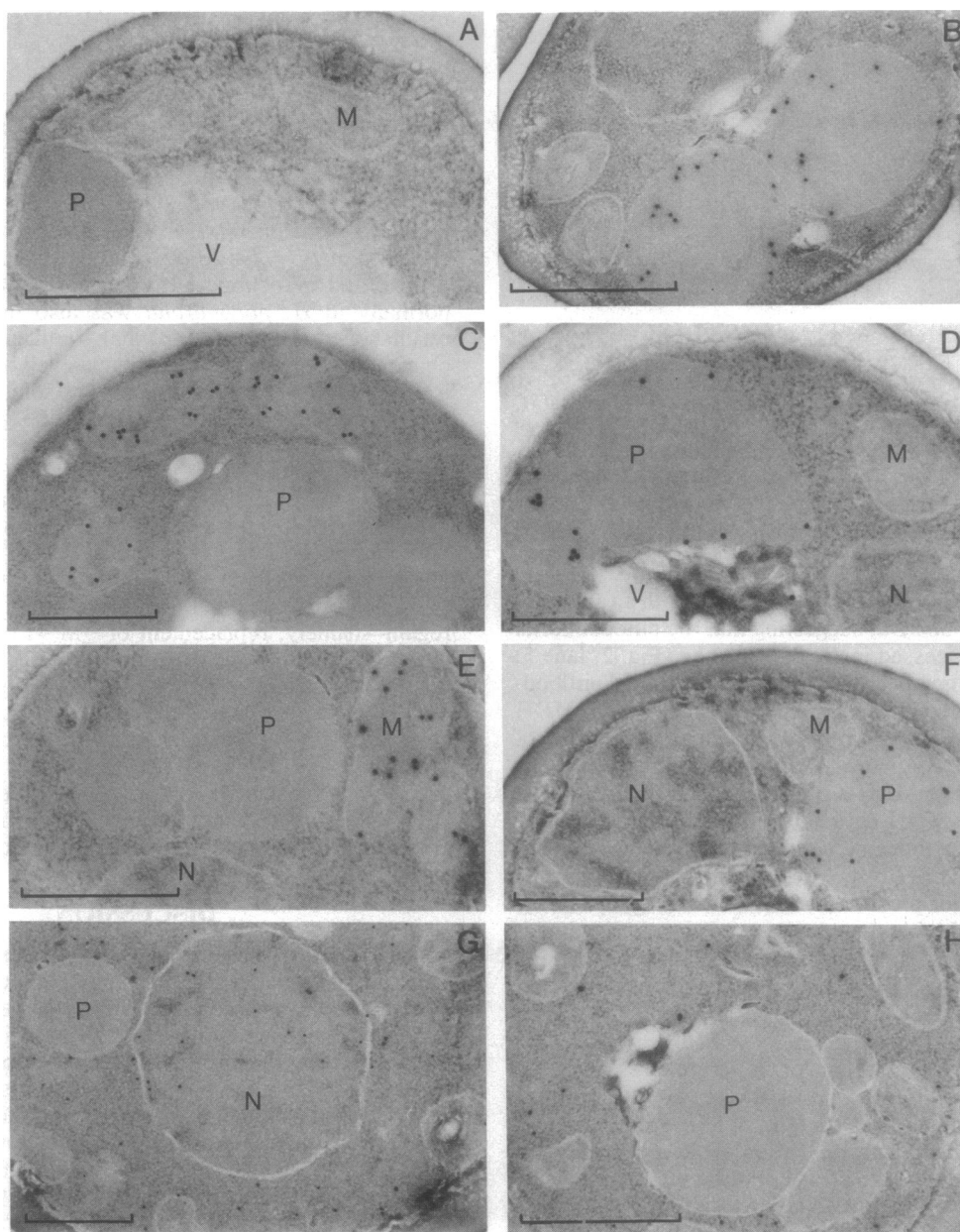


FIG. 4. Electron micrographs prepared from ultrathin sections of aldehyde-fixed cells of *H. polymorpha* transformants, labeled with specific antibodies against gMDH and goat anti-rabbit antibodies conjugated with gold, unless otherwise stated. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. (Bars = 0.5 μm .) (A) Untransformed control cell; peroxisomes and mitochondria lack gold labeling. (B) *H. polymorpha* with pre-gMDH; only peroxisomes are labeled; mitochondria remain unlabeled. (C) Labeling is confined to mitochondria in cells synthesizing watermelon pre-mMDH (α -mMDH). (D–H) Different mutated MDH genes. (D) *H. polymorpha* pre-g::mMDH; peroxisomes are specifically labeled (α -mMDH). (E) *H. polymorpha* with pre-m::gMDH; mitochondria are specifically labeled. (F) *H. polymorpha* with $^{26}\text{EE}\rightarrow\text{LV}$; only peroxisomes are labeled. (G) *H. polymorpha* $^{17}\text{HL}\rightarrow\text{DE}$; specific labeling is on the cytosol and nucleus; peroxisomes show no labeling. (H) Labeling of the cytosol in cells in which mature gMDH is synthesized; both peroxisomes and mitochondria remain unlabeled.

histidine (26). Exchanging ^{17}HL into DE in the gMDH precursor protein severely impeded, but did not fully abolish, cleavage and import in a homologous *in vitro* import system using crude watermelon organelle pellets (27).

Taken together, we have shown that the presequence of gMDH, and specifically the PTS2 motif RI-X₅-HL, is essential for targeting of the protein into microbodies. These results indicate that, apart from PTS1, the PTS2 import machinery might also be highly conserved between lower and higher eukaryotes.

Processing of the glyoxysomal presequence, on the other hand, had not occurred in the yeast and thus is not an essential step during import. This is in line with the findings that in yeast peroxisomal thiolase (9) and MDH (28), the topogenic information is contained in the mature protein.

Also, for enzymatic activity the removal of the glyoxysomal presequence is not necessary, since both wild-type and mutated pre-gMDHs are enzymatically active. This is apparently not the case for the hybrid protein pre-g::mMDH. Additional functions may thus be present in the N-terminal presequences of gMDH and peroxisomal thiolases that are used in higher eukaryotes but not in lower eukaryotes.

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