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Characterization of the *Hansenula polymorpha CPY* Gene Encoding Carboxypeptidase Y

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We have isolated the *Hansenula polymorpha CPY* gene encoding carboxypeptidase Y (Hp-CPY). The deduced amino acid sequence revealed that Hp-CPY consists of 541 amino acids and has a calculated Mr of 60,793. The protein is highly similar to *Saccharomyces cerevisiae* CPY (61·8% identity). At the N-terminus of Hp-CPY signals for the entry into the secretory pathway and subsequent sorting to the vacuole were identified. Immunocytochemically, using monospecific antibodies raised against Hp-CPY, the protein was localized to the vacuole. On Western blots, a diffuse protein band was observed in extracts of *H. polymorpha* cells, suggesting that the protein is glycosylated. This was confirmed by endoglycosidase H treatment, which resulted in a strong reduction of the apparent Mr of the protein. We have investigated the effect of *CPY* deletion on the degradation of peroxisomes, an autophagous process that occurs when the organelles become redundant for growth. In Δ*cpy* cells peroxisomal proteins were degraded in the vacuole as efficiently as in wild-type *H. polymorpha* cells, indicating that CPY is not a major proteinase in this pathway. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — autophagy; glycosylation; peroxisome turnover; methylotrophic yeast; vacuolar proteinases

INTRODUCTION

To date, the vacuolar proteolytic system in the methylotrophic yeast *Hansenula polymorpha* has attracted little attention. In contrast, many proteases that occur in the *Saccharomyces cerevisiae* vacuole have been studied in great detail. The bakers yeast vacuole was shown to contain several proteases: proteinase A (prA); proteinase B (PrB); carboxypeptidase Y (CPY); carboxypeptidase S; aminopeptidase Y, dipeptyl aminopeptidase B and aminopeptidase I (for reviews, see Klionsky *et al.*, 1990; Van den Hazel *et al.*, 1996), proteins that play an important role in protein turnover. From these yeast proteases, CPY has been characterized in detail (see e.g., Valls *et al.*, 1987, 1990). CPY is synthesized as a preproprotein. It contains an

N-terminal signal peptide, which is removed upon entry into the lumen of the endoplasmic reticulum (ER), where the resulting proprotein becomes Asn-glycosylated at four locations. Elongation of these carbohydrate chains occurs during transport of the protein through the Golgi complex. Finally, sorting of the proCPY to the vacuole occurs. This requires the Gln–Arg–Pro–Leu sequence in the pro-region of the protein. In the vacuole, a PrA/PrB-catalysed cleavage of the propeptide of proCPY occurs, a step that is required for proper folding and activation of the enzyme (Winther and Sorensen, 1991; Ramos *et al.*, 1994). The proteases PrA and PrB have also been shown to be essential for the maturation and activation of other vacuolar enzymes (see Klionsky *et al.*, 1990). Hence, *prA prB* mutants are almost devoid of vacuolar proteolytic activity.

Vacuolar proteases play an important role in the degradation of peroxisomes (Tuttle and Dunn, 1995; Chiang *et al.*, 1996). Peroxisomes are cell

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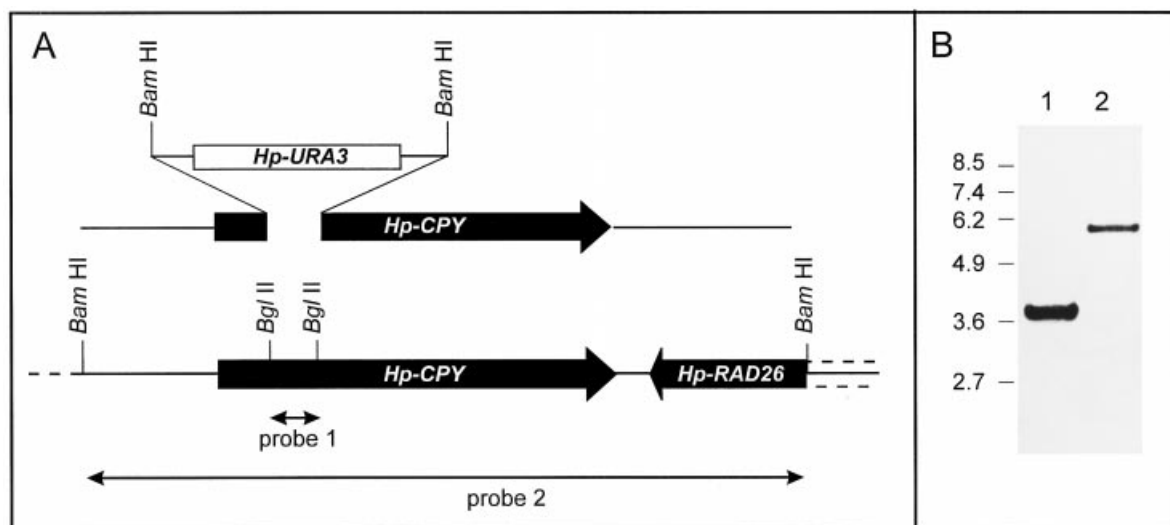
Figure 1.   Disruption of the *H. polymorpha CPY* gene. (A) Schematic representation of the genomic region comprising *CPY*, and deletion of *CPY* by homologous recombination with a 6 kb *Bam*HI fragment containing *H. polymorpha URA3* flanked by *CPY* sequences. Only relevant restriction sites are indicated. The DNA fragments used as probes during this study are also indicated.
   (B) Genomic DNA of WT *H. polymorpha* (lane 1) and Δ*cpy* (lane 2) cells was digested with *Bam*HI and analysed by Southern blotting to demonstrate correct integration of the Δ*cpy* fragment. The 4 kb *Bam*HI fragment of plasmid pBS-CPYa was used as a probe (probe 2 in A). Fragment lengths of the marker, phage SPPI DNA restricted with *Eco*RI, are given in kilobases (kb) to the left of the figure.

organelles surrounded by a single membrane which are present in almost all eukaryotic cells. Characteristic features of these organelles are their functional versatility and their inducibility (Lazarow and Fujiki, 1985). In *H. polymorpha*, peroxisome proliferation is readily induced by a number of carbon and organic nitrogen sources used for growth. Under these conditions the organelles contain key enzymes involved in the metabolism of these substrates (Veenhuis and Harder, 1991). On the other hand, peroxisomes are rapidly degraded when cells are shifted to conditions in which the organelles become redundant for growth. In *H. polymorpha* peroxisomes are degraded individually by an autophagic process involving: (a) sequestration of the organelle to be degraded by a number of membranous layers, presumably derived from the ER; (b) fusion of the sequestered compartment with the vacuole or an autophagic vesicle; and (c) degradation of the peroxisome contents by vacuolar hydrolases (Veenhuis *et al.*, 1983). It has been observed in *S. cerevisiae* and *P. pastoris* that mutants lacking PrA or PrA+PrB activity accumulate undegraded cytosolic proteins and organelles, including peroxisomes, in the vacuole under conditions that induce

autophagy (Tuttle and Dunn, 1995; Chiang *et al.*, 1996). Because *prA prB* mutants lack most vacuolar protease activity, the role of specific proteases in peroxisome degradation was not investigated in these studies. Here we focus on the role of carboxypeptidase Y (CPY) in peroxisome degradation in *H. polymorpha*. We have cloned the gene and studied the effect of *CPY* deletion on selective peroxisome degradation.

MATERIALS AND METHODS

Organisms, media and growth conditions

Hansenula polymorpha NCYC495 and auxotrophic derivatives thereof (Gleeson and Sudbery, 1988) were grown at 37°C in rich medium containing 1% yeast extract, 2% peptone and 1% glucose (YPD), or in selective minimal media containing 0·67% yeast nitrogen base without amino acids (DIFCO), supplemented with 1% glucose (YND) or 0·5% methanol (YNM). Amino acids and uracil were added to a final concentration of 30 μg/ml. *Escherichia coli* DH5α (Sambrook *et al.*, 1989) was grown at 37°C in LB medium supplemented with the appropriate antibiotics.

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Hp-CPY   MKLSMSVLALVATCIGSAQAAAIKKDTAGQHPLGMNSKFGDVFVEKGKSLLDRVS   55
Pp-CPY   MILHTYIILSLLTIFPKAIGLSL-----QMPMALEASYA--------SLVEKAT    41
Sc-CPY   MKAFTSLLCGLGLSTTLAKAISL-----QRPLGLDK---DVLLQAAEKF--GLD    44
           *      ..  .       *    ..     * *..                .

Hp-CPY   EVVSESSKNISPELRDIWNEMEAKFPDTLKNMKLKSEPVVKITKKPADFWDFNVF   110
Pp-CPY   LAVGQEIDAIQKGIQQGWLEVETRFPTIVSQLSYSTGPKFAIKKKDATFWDFYVE    96
Sc-CPY   LDLDHLLKELDSNVLDAWAQIEHLYPNQVMSLETSTKPKFPEAIKTKKDWDFVVK    99
           .        .  .    *   .*  .*  .    .*       *     *** *

Hp-CPY   NEKFSNYKLRVKK-TDPGALGLD-HTRQYSGYLDVEDEDKHFFYWMFESRNDPAN   163
Pp-CPY   SQELPNYRLRVKR-NNPEVLKVD-FTKQYSGYLDVEADDKHFFYWFFESRNDPQN   149
Sc-CPY   NDAIENYQLRVNKIKDPKILGIDFNVTQYTGYLDVEDEDKHFFFWTFESRNDPAK   154
           ** ***  .    *   * .*   **.****** .*****.* *******

Hp-CPY   DPVILWLNGGPGCSSLTGMLFELGSASIGPDLKPINNPYSWNSNATVIFLDQPVN   218
Pp-CPY   DPIILWLNGGPGCSSLTGLFFELGSSRINENLKPIFNPYSWNGNASIIYLDQPVN   204
Sc-CPY   DPVILWLNGGPGCSSLTGLFFELGPSSIGPDLKPIGNPYSWNSNATVIFLDQPVN   209
           **.***************. ****  .*    **** ****** **..*.******

Hp-CPY   VGFSYSSKS-VSNTVAAGKDVYAFLELFYQQFPHLLKN--DFHIAGESYGGHYIP   270
Pp-CPY   VGFSYSSSS-VSNTVVAGEDVYAFLQLFFQHFPEYQTN--DFHIAGESYAGHYIP   256
Sc-CPY   VGFSYSGSSGVSNTVAAGKDVYNFLELFFDQFPEYVNKGQDFHIAGESYAGHYIP   264
           ******  * ***** ** *** ** **.  **     ********* *****

Hp-CPY   VFASEILTHADRSFNLTSVLIGNGLTDPLNQYPFYERMACSTDGGYEPTLDESEC   325
Pp-CPY   VFADEILSQKNRNFNLTSVLIGNGLTDPLTQYRYYEPMACG-EGGAPSVLPADEC   310
Sc-CPY   VFASEILSHKDRNFNLTSVLIGNGLTDPLTQYNYYEPMACG-EGGEPSVLPSEEC   318
           *** ***.    * **************** ** .** *** .**    *    **

Hp-CPY   EGMLETLPRCLSLIESCYSSQSVFSCVPASIYCNNAQLGPFQKTGRNVYDVRKMC   380
Pp-CPY   ENMLVTQDKCLSLIQACYDSQSAFTCAPAAIYCNNAQMGPYQRTGKNVYDIRKEC   365
Sc-CPY   SAMEDSLERCLGLIESCYDSQSVWSCVPATIYCNNAQLAPYQRTGRNVYDIRKDC   373
             *   . .** ** .** ***  .* ** .*******. *.*.**.****.** *

Hp-CPY   EG-TLCYKDMEYIDQYLNQDFVKEKIGAEVETYESCNFDVNRNFLFAGDWMKPYH   434
Pp-CPY   DGGSLCYKDLEFIDTYLNQKFVQDALGAEVDTYESCNFEINRNFLFAGDWMKPYH   420
Sc-CPY   EGGNLCYPTLQDIDDYLNQDYVKEAVGAEVDHYESCNFDINRNFLFAGDWMKPYH   428
           .* ***   .  ** **** .* . .****. *.*.**.****.**************

Hp-CPY   KNVINLLEQGLPVLIYAGDKDFICNWLGNQAWSNELPWSGHEEFESAELYNLTL-   488
Pp-CPY   EHVSSLLNKGLPVLIYAGDKDFICNWLGNRAWTDVLPWVDADGFEKAEVQDW-L-   473
Sc-CPY   TAVTDLLNQDLPILVYAGDKDFICNWLGNKAWTDVLPWKYDEEFASQKVRNWTAS   483
             *  **   **.*.************** **. ***   .*      .

Hp-CPY   KDGTKVGEVKNAGKFTFARMFDGGHMVPYDQPESSLAMVNRWIAGDYSLGTKK    541
Pp-CPY   VNGRKAGEFKNYSNFTYLRVYDAGHMAPYDQPENSHEMVNRWISGDFSFH      523
Sc-CPY   ITDEVAGEVKSYKHFTYLRVFNGGHMVPFDVPENALSMVNEWIHGGFSL       532
           ** *     **. *.. *** *.* **    *** ** * .*
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Figure 2.   Alignment of yeast carboxypeptidase Y sequences. The primary sequences of carboxypeptidase Y from *H. polymorpha* (Hp-CPY), *P. pastoris* (Pp-CPY; Ohi *et al.*, 1996) and *S. cerevisiae* (Sc-CPY; Valls *et al.*, 1987) were aligned using the CLUSTAL program. Gaps were introduced to maximize the similarity. Identical residues are indicated by an asterisk below the sequences, conservative replacements by a dot. Putative pre-sequences, vacuolar targeting signals, N-linked glycosylation sites, and serine and histidine active site residues are indicated in white letters on a black background. The putative pro-sequences of the proteins are indicated by the black line above the sequences.

DNA procedures

   H. polymorpha cells were transformed using the electroporation method (Faber *et al.*, 1994). Recombinant DNA manipulations were as described (Sambrook *et al.*, 1989). Southern blot analysis and colony hybridization were performed using the ECL direct nucleic acid labelling and detection system (Amersham Corp., Arlington Heights, IL). Biochemicals were obtained from Boehringer, Mannheim, Germany.

Cloning and sequence analysis of the CPY gene

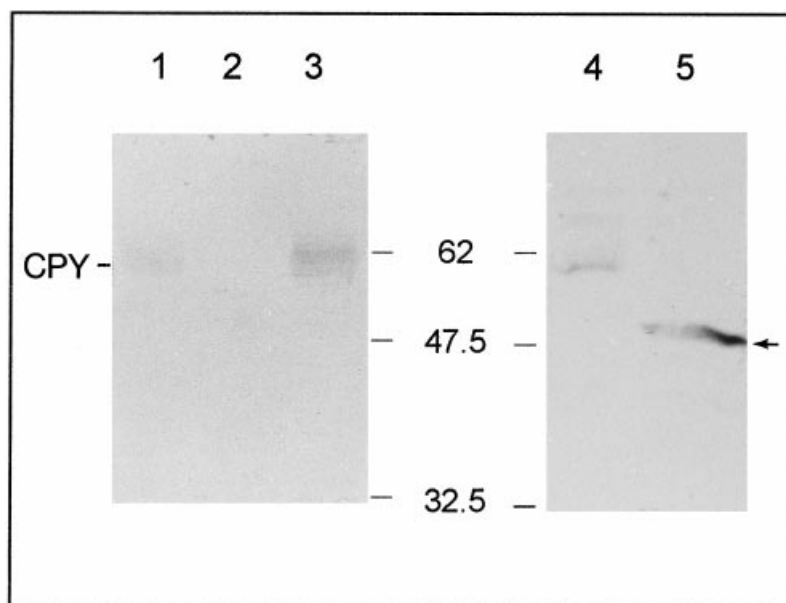   To isolate the *CPY* gene, a 260 bp *H. polymorpha Bgl*II fragment was used as a probe to

Figure 3. Immunological detection of CPY protein. Western blot of whole-cell lysates of *H. polymorpha* WT (lane 1). Δ*cpy* (lane 2) and Δ*cpy*[PYT3-CPY] (slightly over-expressing *CPY*; lane 3) cells grown in batch culture on YND medium. The very weak, diffuse protein band reflects the low level of CPY protein in cells growing on glucose. Equal amounts of protein (20 μg) were loaded in each lane. Lanes 4 and 5 show whole cell lysates (30 μg of protein) of YND-grown *H. polymorpha* WT cells after SDS-denaturation (lane 4), followed by endoglycosidase H treatment (lane 5). The blots were decorated with α-CPY antibodies. Protein sizes of the marker are indicated in kDa. The arrow indicates deglycosylated CPY at approximately 47 kDa.

screen a *H. polymorpha* genomic DNA library constructed in vector pYT3 (Tan *et al.*, 1995), using bacterial colony hybridization. Five positive clones were isolated. One of these clones, designated pYT3-CPY, which contained an insert of about 5 kb that hybridized to the probe, was analysed further. A *Bam*HI fragment of approximately 4 kb was subcloned in both orientations into *Bam*HI-digested pBluescript II SK$^+$ (Stratagene, San Diego, CA) to create plasmids pBS-CPYa and pBS-CPYb. Double-stranded DNA sequencing of a 2·5 kb region of the subclones was carried out on an ABI 313A automatic sequencer (Applied Biosystems Inc.) using the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit. In addition, several selected oligonucleotides were synthesized to complete or confirm certain portions of the DNA sequence. For analysis of the DNA and amino acid sequences, the PC-GENE program release 6.70 (IntelliGenetic Inc., Mountain View, CA) was used. The TBLASTN algorithm (Altschul *et al.*, 1990) was used to screen

databases at the National Center for Biotechnology Information (NCBI, Bethesda, MD). The nucleotide sequence of *H. polymorpha CPY* was deposited at GenBank and was assigned Accession No. AF085063.

Construction of a CPY deletion mutant

To disrupt the wild-type *CPY* gene, a 2·3 kb *Bam*HI fragment containing the *H. polymorpha URA3* gene (Merckelbach *et al.*, 1993) was ligated into *Bgl*II-digested pBS-CPYa, resulting in a deletion of nt 639–904 of *CPY* encoding amino acids 195–282 (Figure 1A). The resulting plasmid was digested with *Bam*HI top yield a 6 kb linear fragment containing the *URA3* gene flanked by *CPY* sequences (Figure 1A), which was transformed to *H. polymorpha* NCYC495 (*leu1.1 ura3*). Uracil prototrophic transformants were examined for the proper integration of the *URA3* gene at the *CPY* locus by Southern blot analysis (Figure 1B). The resulting putative Δ*cpy* strains were also tested for CPY activity.
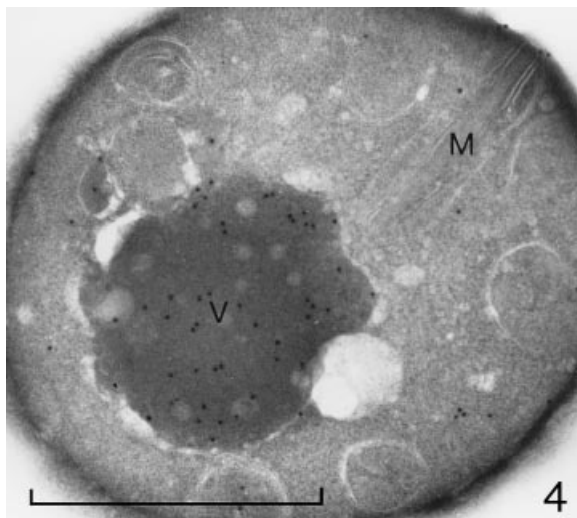
Figure 4. Immunocytochemical localization of CPY in *H. polymorpha*. *H. polymorpha* WT cells were grown on YND to the stationary phase. Cryosections were prepared and labelled with α-CPY antibodies. M, mitochondrion; V, vacuole. Bar indicates 1 μm.

Generation of anti-CPY antibodies

The Protein Fusion and Purification System (New England Biolabs, Beverly, MA) was used for the production of a maltose-binding protein (MBP)-CPY fusion protein in *E. coli*. A 2·2 kb *Bgl*II–*Pst*I fragment encoding amino acids 283–541 of CPY was cloned in frame behind the *malE* gene between the *Bam*HI and *Pst*I sites of the pMAL-c2 vector. Expression of the *malE–CPY* chimeric gene was induced by the addition of 0·3 mM IPTG to exponentially growing cultures. The fusion protein was recovered by centrifugation as inclusion bodies, which were used for immunization in rabbit (Harlow and Lane, 1988).

Biochemical methods

Crude extracts were prepared as described (Waterham *et al.*, 1994), except that 1 mM phenylmethylsulphonylfluoride and 2·5 μg/ml of leupeptine were added. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as standard. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described (Laemmli, 1970). Western blotting was performed using either the Protoblot immunoblotting system (Promega Biotec) or the ECL system (Amersham Corp., Arlington Heights, IL). Alcohol oxidase (Verduyn *et al.*, 1984), catalase (Lück, 1963) and

CPY (Jones, 1991) activities were determined in cell extracts by established procedures. CPY activity in *H. polymorpha* colonies was detected using the FAST-Garnet overlay technique (Jones, 1991). Deglycosylation of CPY in cell extracts was performed using endoglycosidase H, as described (Elgersma *et al.*, 1997). Degradation of peroxisomal proteins in batch cultures of *H. polymorpha* was followed by enzyme activity measurements and Western blotting using extracts of methanol-grown cells shifted to 0·5% glucose (Titorenko *et al.*, 1995).

Electron microscopy

H. polymorpha cells were fixed in 3% formaldehyde in 50 mM Na-phosphate buffer (pH 7·2). Cryosections were prepared according to Tokuyasu (1973) and immunolabelled using α-CPY antibodies.

RESULTS AND DISCUSSION

Isolation and characterization of the H. polymorpha CPY gene

To clone the *H. polymorpha CPY* gene, we used a genomic 260 bp *Bgl*II *H. polymorpha* fragment, which showed high similarity to *S. cerevisiae PRC1* encoding carboxypeptidase Y (Valls *et al.*, 1987) as a probe (probe 1 in Figure 1A). This fragment had become available from an earlier screen for suitable *H. polymorpha* promoters (P. E. Sudbery, unpublished data). In Southern blot analyses this fragment strongly hybridized with *H. polymorpha* genomic DNA (data not shown). Subsequently, we screened a genomic *H. polymorpha* gene library with this probe. One positive clone, designated pYT3-CPY, containing a hybridizing insert of approximately 5 kb, was selected for further analysis. The nucleotide sequence of a 2509 bp region of this clone was determined. Sequence analysis revealed the presence of one complete and one incomplete open reading frame (ORF; see Figure 1A). The complete ORF encoded a protein of 541 amino acids with a calculated Mr of 60,793, which was highly similar to CPY from *S. cerevisiae* (Sc-CPY; Valls *et al.*, 1987) and *P. pastoris* (Pp-CPY; Ohi *et al.*, 1996) (62·4% and 60·4% identity, respectively; Figure 2). Therefore, we concluded that ORF1 represents the *H. polymorpha CPY* gene. The incomplete ORF2, which showed similarity to the *S. cerevisiae*
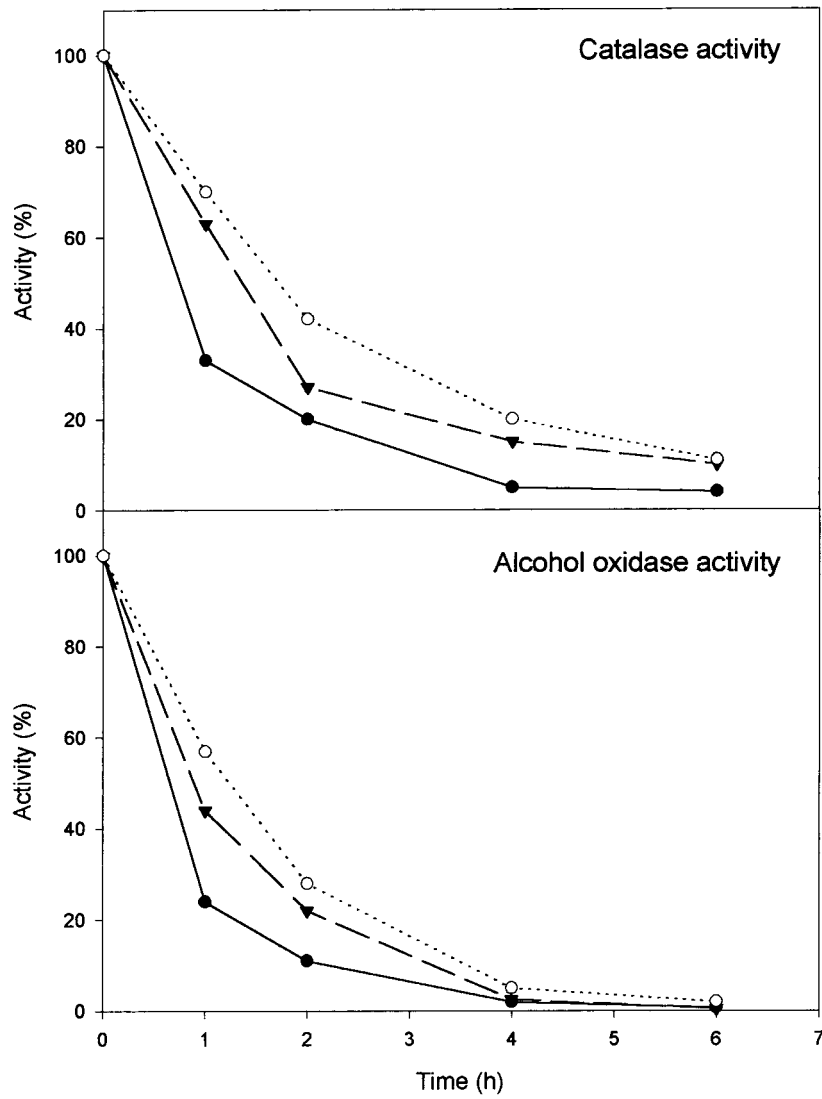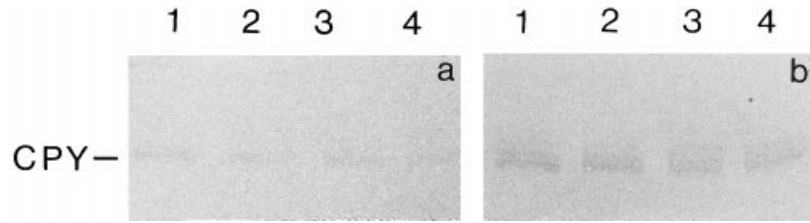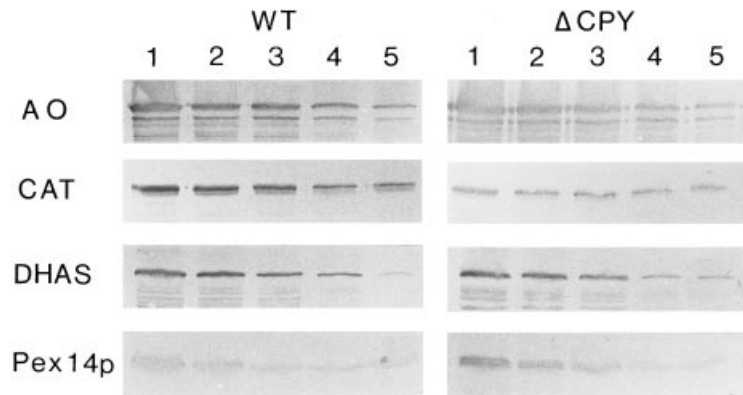
Figure 5.   (A) and (B).

Figure 5.   (C)

Figure 5.   Selective inactivation of peroxisomes in *H. polymorpha*. (A) Levels of CPY protein in crude extracts of WT *H. polymorpha* (a) and Δ*cpy*[pYT3-CPY] (b) during glucose-induced peroxisome degradation. Samples were taken at 0 h (lane 1), 1 h (lane 2), 3 h (lane 3) and 5 h (lane 4) after the addition of 0·5% glucose to cells exponentially growing on methanol. (B) Kinetics of changes in the specific enzyme activities of catalase and alcohol oxidase, determined in crude extracts from WT (closed circles), Δ*cpy* (closed triangles) and Δ*cpy*[pYT3-CPY] (open circles) cells after the addition of 0·5% glucose to batch cultures growing exponentially on methanol. The decrease in specific activities is expressed as the percentage of the initial value, which is set to 100%. Data are corrected for growth of the cells on glucose. (C) Decrease in the level of the peroxisomal matrix enzymes alcohol oxidase (AO), catalase (CAT), dihydroxyacetone synthase (DHAS) and the peroxisomal membrane-associated protein Pex14p in whole cell lysates from WT *H. polymorpha* and Δ*cpy* cells in the initial hours after the shift of cells from methanol to glucose. Samples were taken at 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4) and 6 h (lane 5) after the addition of glucose. Equal amounts of protein were loaded per lane. Identical results were obtained with cells from Δ*cpy*[pYT3-CPY] (not shown).

RAD26 gene (Van Gool *et al.*, 1994), and presumably represents its *H. polymorpha* orthologue, was not studied further.

The deduced primary sequence of *H. polymorpha* CPY (Hp-CPY) contains a putative presequence involved in import of the protein into the ER (cf. Klionsky *et al.*, 1990). This signal extends from amino acid 1 to the alanine at position 20 (Figure 2). A putative vacuolar targeting signal consisting of amino acids 30–33 (Gln–His–Pro–Leu) similar to that observed in Sc-CPY (Gln–Arg–Pro–Leu), was also evident. Additionally, a putative pro-region that shows only little conservation to Sc-CPY and Pp-CPY is located between amino acids 21 and 122. Thus we calculate that the mature Hp-CPY protein is presumably composed of 419 amino acids with a predicted Mr of 47,142. In contrast to Sc-CPY, which contains four N-glycosylation sites, Hp-CPY is predicted to contain only three sites. These sites correspond to three of the four locations in Sc-CPY. As expected,

the active site Ser and His residues are conserved in Hp-CPY (Figure 2).

Construction and characterization of a CPY disruption mutant

To study the possible role of CPY in selective peroxisome degradation in *H. polymorpha*, a *CPY* disruption strain was constructed. We found that the CPY activity in the Δ*cpy* strain was reduced significantly as compared to the wild-type (0·2 vs 3·8 mU/mg protein). Polyclonal antibodies were raised against a MBP–CPY fusion protein in rabbit. In order to test the specificity of the antiserum (α-CPY), Western blots were prepared from crude extracts of YND-grown *H. polymorpha* WT, Δ*cpy* and Δ*cpy*[pYT3-CPY] cells (Figure 3, lanes 1–3). On the blots a diffuse protein band of approximately 60 kDa could be observed in WT and Δ*cpy*[pYT3-CPY] extracts, which was absent in Δ*cpy* extracts. From these results we conclude

that the antiserum specifically recognizes *H. polymorpha* CPY. The larger size of the protein as compared to its calculated molecular mass (47 kDa), as well as its diffuse pattern on Western blots, indicates that Hp-CPY is presumably modified by N-linked glycosylation in the ER followed by mannosylation in the Golgi complex. This was confirmed by treatment of a WT cell extract with endoglucosidase H, which cleaves N-linked glycans from proteins. After deglycosylation, α-CPY antibodies recognized an approximately 47 kDa protein, instead of the 60 kDa protein (Figure 3, lanes 4 and 5).

The α-CPY antiserum was subsequently used for the localization of CPY by immunocytochemical methods. Labelling of cryosections of stationary YND-grown WT *H. polymorpha* cells with the antiserum resulted in the specific labelling of vacuolar profiles (Figure 4). Labelling of other cell compartments was not observed, suggesting that Hp-CPY is a constituent of the vacuole. No labelling was observed in Δ*cpy* controls (data not shown).

Growth experiments indicated that deletion of the *CPY* gene did not significantly affect growth of *H. polymorpha* on glucose and methanol. Also, no major differences in the protein levels of the major peroxisomal enzymes alcohol oxidase (AO), catalase (CAT) and dihydroxyacetone synthase (DHAS) were observed between Δ*cpy* and WT cell extracts (data not shown). Additionally, peroxisome proliferation in these cells was virtually unaffected, suggesting that deletion of *CPY* did not affect the synthesis of normal peroxisomes in *H. polymorpha*.

Role of CPY in glucose-induced selective peroxisome degradation

As described before, peroxisomal enzymes in methanol-grown *H. polymorpha* are susceptible to carbon catabolite inactivation. In order to study the role of CPY in the peroxisome degradation process, we analysed the fate of these organelles during the initial hours after the addition of 0·5% glucose to methanol-grown WT, Δ*cpy* and Δ*cpy*[pYT3-CPY] cells. This addition did not change the level of CPY protein in cell extracts of WT and Δ*cpy*[pYT3-CPY] cells during subsequent growth (Figure 5A). Enzyme activity measurements revealed that the patterns of inactivation of the major peroxisomal enzymes AO and CAT after addition of glucose were largely identical for the

three strains (Figure 5B). Western blot analysis of crude extracts prepared from these cells revealed that the inactivation of AO, DHAS and CAT was due to a decrease in the levels of AO, DHAS and CAT protein in the cells (Figure 5C). As demonstrated before, the data confirmed that inactivation of peroxisomal enzymes (most likely by modification) precedes the proteolytic degradation of these proteins (Bruinenberg *et al.*, 1982). Identical degradation patterns were found for Pex3p, an integral component of the peroxisomal membrane, and Pex14p, a membrane-associated protein (Figure 5C, shown for Pex14p). These results suggest that in *H. polymorpha* CPY is not the major protease involved in the proteolytic degradation of peroxisomal constituents during selective degradation of the organelle.

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