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Targeting signal of the peroxisomal catalase in the methylotrophic yeast *Hansenula polymorpha*

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The methylotrophic yeast, *Hansenula polymorpha*, harbours a unique catalase (EC 1.11.1.6), which is essential for growth on methanol as a carbon source and is located in peroxisomes. Its corresponding gene has been cloned and the nucleotide sequence determined. The deduced amino acid sequence displayed the tripeptide serine-lysine-isoleucine at the extreme C-terminus, which is similar to sequences of other peroxisomal targeting signals. Exchange of the ultimate amino acid, isoleucine, of catalase for serine revealed a cytosolic enzyme activity and a concomitant loss of peroxisome function. We concluded that the tripeptide is essential for targeting of catalase in *H. polymorpha*.

Microbody; Peroxisome; Protein sorting; Targeting signal; *Hansenula polymorpha*

1. INTRODUCTION

Peroxisomes are common organelles in eukaryotic cells harbouring various metabolic functions [1,2]. Import of peroxisomal proteins occurs post-translationally. Their sorting requires specific targeting signals within the primary sequence. In mammalian cells a tripeptide of amino acids, S-K-L, at the C-terminus was recently shown to be both necessary and sufficient for import [3,4]. Although this signal is widespread in eukaryotes [3] numerous peroxisomal proteins are devoid of such a sequence. Furthermore, internal targeting signals of as yet undefined nature were proposed to be present in yeasts [5,6]. Peroxisomal thiolases were recently shown to contain targeting signals within a cleavable leader sequence at the N-terminus [7,8].

We show here that the targeting signal of the peroxisomal catalase in the methylotrophic yeast, *Hansenula polymorpha*, also resides at the C-terminus. Its sequence of amino acids, S-K-I-COOH, suggests that the S-K-L motif is less conserved than originally reported [3].

2. MATERIALS AND METHODS

2.1. *Strains and media*

Amplification of plasmids was done in *Escherichia coli* MB 1000 (*hsrK hsmK lac trp pyrF*) [9] grown in either LB supplemented with 100 μg ampicillin/ml or in M9 medium [10] with 20 μg tryptophan. Yeast transformation was done either with strain LR9 (*odc1*), an orotidine-5'-phosphate decarboxylase-deficient mutant [11] of *H. polymorpha* (ATCC 34438) as well as with HM1-39 (*odc1 leu2*), or with the catalase-deficient mutant, INT-13 (*odc1 leu2 ctt1::LEU2*), which is unable to grow on methanol as a carbon source. Yeast cells were grown on 0.67% Yeast nitrogen base (Difco) without amino acids supplemented with either 1% methanol or 2% glycerol (de-repressing conditions) as carbon source. If required, uracil or leucine was added at 20 μg/ml.

2.2. *Plasmid constructions*

For constructing pHCAT-B the vector pHARS1 [11] was cut with *Cla*I and, after the ends were made blunt by Klenow polymerase, an 8mer *Xho*I linker was inserted. A 2.4 kb *Sal*I/*Eco*RV fragment encoding the catalase gene (position 1–2308; see Fig. 3) was inserted into the created *Xho*I site of pHARS1 after ligation of a further 8mer *Xho*I linker to the *Eco*RV site of the catalase fragment. For construction of plasmid pHCAT-K a *Pst*I/*Eco*RV (position 983–2308; see Fig. 3) subclone of the catalase gene was inserted into the multi-cloning site of the plasmid pSELECT^{-1} of the Altered Sites in vitro Mutagenesis System (Promega). Oligonucleotide-directed mutagenesis was performed according to the description of the manufacturer. The oligonucleotide with the sequence 5'-CGATGATTATTTTTGGATGG was used to alter the last triplet ATA (isoleucine) of the coding region into AAA (lysine). After mutagenesis the altered gene fragment was recloned into corresponding sites of the vector pHCAT-B to generate the plasmid pHCAT-K. The correct base change was verified by DNA sequencing.

2.3. *Gene replacement*

Strain HM1-39 was transformed with a linear 4.2 kb restriction fragment that carried the LEU2 gene of *Saccharomyces cerevisiae* as a 2.3 kb *Xho*I/*Sal*I fragment [12] inserted into the catalase gene, eliminating the *Ball*/*Cla*I fragment between base pairs 1264 and 2172 (see Fig. 3). Thus, the truncated catalase lacked more than half of the coding region corresponding to 301 amino acids at the C-terminus. Exchange of the truncated catalase gene with the genomic gene in HM1-39 by recombination was verified by Southern analysis (Fig. 1).

2.4. *DNA sequencing*

The dideoxynucleotide chain-terminating procedure [13,14] was used with a T7 polymerase sequencing kit (Pharmacia). The protocol of the manufacturer was followed. Both DNA strands were sequenced.

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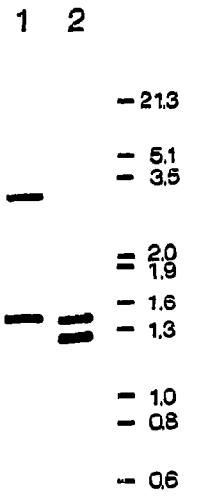

Fig. 1. Southern analysis of catalase gene replacement in *H. polymorpha*. Chromosomal DNA was cut with *Eco*RI and *Bgl*II, respectively. Restriction fragments were probed with a *Sal*I–*Cla*I fragment spanning bp 1–2,172 (see Fig. 3) using the digoxigenin labeling kit (Boehringer). The *Eco*RI/*Bgl*II fragment of 1,286 bp (position 819–2,105, see Fig. 3) in HM1-39 (lane 2) was shifted to a size of greater than 2,745 bp (inserted LEU2 gene plus the 455 bp *Eco*RI/*Bal*I fragment from position 819–1,264 of the catalase gene, see Fig. 3), i.e. approximately to 3 kb in INT-13 (lane 1). See section 2.3 for further details. DNA standards of molecular mass are given in kb.

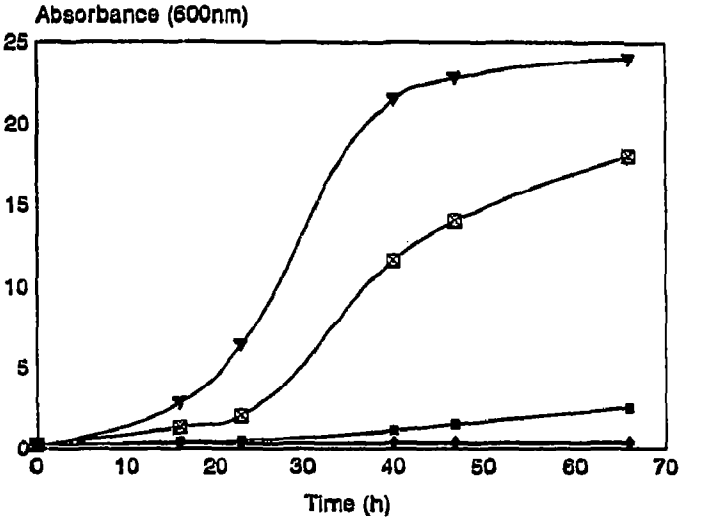

Fig. 2. Growth curve of catalase transformants of *H. polymorpha* on methanol as a carbon source. Cells were pre-grown selectively in Yeast nitrogen base with 1% glucose and then transferred at zero time to 0.2% yeast extract, 0.4% peptone and 2% methanol as a carbon source. Growth was monitored by measuring the optical density at 600 nm. (▼) Wild-type; (□) INT1-13/pCAT-B; (■) INT1-13/pCAT-K; (♦) INT1-13.

2.5. *Cell fractionation*

Transformed yeast cells were grown in Yeast nitrogen base plus 2% glycerol as a carbon source for 36–48 h. Cells (1 ml of culture medium) were converted to protoplasts and gently lysed by osmotic shock essentially as described earlier [15]. The obtained cell lysates were subjected to sucrose gradient centrifugation [15]. Catalase activity was measured spectrophotometrically at 240 nm by H_2O_2 degradation [16].

Crude extracts were obtained by disruption of glycerol-grown yeast cells in a Braun homogenizer (Braun, Melsungen). Cell debris were removed by centrifugation at 10,000 × *g* for 20 min.

3. RESULTS AND DISCUSSION

The gene coding for the unique catalase in *H. polymorpha* was cloned as a 7.5 kb fragment (inserted in the *Bam*HI site of vector pHARS1) from a genomic library by hybridisation to the catalase T gene from *Saccharomyces cerevisiae* [17]. The fragment conferred high catalase activity in the catalase-negative mutant, INT-13, of *H. polymorpha* and restored its ability to grow on methanol as a carbon source (Fig. 2). The nucleotide sequence of a 2,620 bp subclone was determined. It contained one large open reading frame of 1,521 bp corresponding to 507 amino acids (Fig. 3) with high homologies to catalases of various origins (not shown). The C-terminal tripeptide with the sequence S-K-I-COOH appeared to be highly similar to the well-defined S-K-L-COOH motif with respect to sequence homology and location within the polypeptide.

In order to test a functional role of the extreme C-terminus of catalase the ultimate amino acid isoleucine was converted into a lysine by means of site-directed mutagenesis. Transformation of pCAT-K harbouring the mutated gene into strain INT-13 resulted in catalase activity under de-repressed conditions, i.e. with glycerol as a carbon source (Table I). The activity could not restore growth on methanol that requires functional peroxisomes, in contrast to glycerol as a carbon source (Fig. 2). This result, and our previous findings that cytosolic catalase activity did not complement a lack of peroxisomal catalase [15], suggested that the catalase was not targeted to peroxisomes. Indeed, cell fractionation studies revealed that the catalase activity and immuno-blotted catalase protein [15] appeared mostly in the supernatant fraction after centrifugation of cell lysates from pCAT-K transformants, whereas in the case of wild-type cells and pCAT-B transformants regular levels [18] of about 30–40% of catalase activity were pelleted (data not shown). These results were confirmed by sucrose gradient centrifugation of whole cell lysates. Virtually all of the catalase appeared on top of the

Table I

Activities of catalase in crude extracts of *Hansenula polymorpha* transformants

Strain	Plasmid	Specific activity (μmol/min/mg protein)
Wild-type	–	456
INT1-13	–	0
INT1-13	pHCAT-B	188
INT1-13	pHCAT-K	152

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   1  GTCGACGGTGTAAACAGCCTGCAGCTCGCTGGGCTTGAGCGTCGGGTCGGGCTGGAGCGAGCTCTGTGCA
  71  ATGCGGGCCTGTTTTGCCGAGCAAAATGGGATCCAGTACGATCTAGACGGTCAGACAACAACAATGACGA
 141  CTACTTACAGTTTGTGAGAGAACACCATAAGAAAAAGATTGTTGCACAGCCCGCTTTGAGAAGGAAATAA
 211  CCGTAGCCTATATCAGTCACTCCTCGCACCGCAACGATTCTGAAATGATTGCGATTTCCTAATCCAGCCT
 281  TACAAATTCAAGCAACCCAATTGGCCCCACAAACGCAGCAAGTCCAAAAAATGTGCTCGACAAGTTTGTG
 351  GATTTTGCTGTACCGCGGCGGGTGAACGATGCAAAGTGCTGTTTCTTGAAGACAATTGGTCATCAGCATT
 421  CAGCATTCAGCATGGATCTCGTGATCGGAGAGCAACTGTAGAGTGACTCTTATTAATTATGCAACCAAGT
 491  CCAGAAACTCTCCTCAGATATGTCAGCAATTTTGAAACTAGTAAAATAATAGTCTATGCATGCAGGCGGG
 561  GGCAACAGTACTAATTGAAGCCTAAGCAAAAATATATAAAGGCTCCCGGCCGCACCCCAACTTGGCTAAT
 631  CACTGTTGAACAAAATGTCAAACCCCCCTGTTTTCACTACTTCGCAAGGTTGTCCCGTGTCGGATCCCTT
   1                  M  S  N  P  P  V  F  T  T  S  Q  G  C  P  V  S  D  P  F
 701  CACCACCCAACGGATCCCTCTGGATTCCACGGGCTACAAGTATGCCCCTCCAATTGGTCCATTGCTCTTG
  20   T  T  Q  R  I  P  L  D  S  T  G  Y  K  Y  A  P  P  I  G  P  L  L  L
 771  CAGGACTTCAAGCTCATTGACACACTCTCCCACTTTGACAGAGAAAGAATTCCAGAGAGAGTGGTGCACG
  43  Q  D  F  K  L  I  D  T  L  S  H  F  D  R  E  R  I  P  E  R  V  V  H  A
 841  CCAAGGGTGCCGGAGCTTACGGTGTGTTTGAAGTGACCGACGACATTACCGATGTTTGCAGTGCCAAGTT
  67    K  G  A  G  A  Y  G  V  F  E  V  T  D  D  I  T  D  V  C  S  A  K  F
 911  CCTCGACACTGTTGGTAAGAAGACAAGAATTTTCACCAGATTCTCCACTGTGGGTGGAGAAAAAGGTTCT
  90   L  D  T  V  G  K  K  T  R  I  F  T  R  F  S  T  V  G  G  E  K  G  S
 981  GCAGACACAGCCAGGGACCCAAGGGGATTTGCTACCAAATTCTACACAGAAGACGGAAACTTGGACCTTG
 113  A  D  T  A  R  D  P  R  G  F  A  T  K  F  Y  T  E  D  G  N  L  D  L  V
1051  TGTACAACAACACGCCGATCTTCTTCATCAGGGACCCAATTAAGTTCCCCCACTTTATTCACACGCAGAA
 137    Y  N  N  T  P  I  F  F  I  R  D  P  I  K  F  P  H  F  I  H  T  Q  K
1121  GAGAAACCCGGCCACCAACTTGAAGGACCCAAACATGTTCTGGGACTACCTGACGGCCAATGACGAGTCT
 160   R  N  P  A  T  N  L  K  D  P  N  M  F  W  D  Y  L  T  A  N  D  E  S
1191  TTGCACCAAGTCATGTATTTATTCTCCAACAGAGGTACGCCTGCCTCGTACAGAACCATGAACGGCTACT
 183  L  H  Q  V  M  Y  L  F  S  N  R  G  T  P  A  S  Y  R  T  M  N  G  Y  S
1261  CTGGCCACACCTACAAATGGTACAATTCCAAGGGCGAGTGGGTGTATGTGCAGGTCCACTTCATTGCCAA
 207    G  H  T  Y  K  W  Y  N  S  K  G  E  W  V  Y  V  Q  V  H  F  I  A  N
1331  CCAAGGCGTGCACAATCTGCTCGACGAGGAAGCTGGAAGGCTGGCTGGAGAAGATCCAGACCACTCCACC
 230   Q  G  V  H  N  L  L  D  E  E  A  G  R  L  A  G  E  D  P  D  H  S  T
1401  AGAGACTTGTGGGAAGCAATTGAGAAAGGAGATTATCCATCGTGGGAGTGCTACATCCAGACCATGACCC
 253  R  D  L  W  E  A  I  E  K  G  D  Y  P  S  W  E  C  Y  I  Q  T  M  T  L
1471  TTGAGCAGTCCAAGAAGCTGCCATTCTCTGTGTTTGACTTGACCAAGGTCTGGCCACACAAGGACTTCCC
 277    E  Q  S  K  K  L  P  F  S  V  F  D  L  T  K  V  W  P  H  K  D  F  P
1541  GCTGAGACACTTTGGCAGATTCACTCTTAATGAGAATCCTAAGAATTACTATGCTGAAACGGAGCAGATT
 300   L  R  H  F  G  R  F  T  L  N  E  N  P  K  N  Y  Y  A  E  T  E  Q  I
1611  GCCTTCTCTCCTTCGCACACCGTTCCTGGAATGGAGCCTTCGAACGACCCTGTCTTGCAATCGAGACTGT
 323  A  F  S  P  S  H  T  V  P  G  M  E  P  S  N  D  P  V  L  Q  S  R  L  F
1681  TTTCGTACCCAGACACGCACAGACACAGACTTGGACCTAACTACCACCAGATCCCTGTCAACTGTCCACT
 347    S  Y  P  D  T  H  R  H  R  L  G  P  N  Y  H  Q  I  P  V  N  C  P  L
1751  GAAATCGGGCTCGTTCAATCCTATCAACAGAGACGGTCCTATGTGTGTTGATGGCAACTTGGGAGGCACG
 370   K  S  G  S  F  N  P  I  N  R  D  G  P  M  C  V  D  G  N  L  G  G  T
1821  CCGAACTACGCAAACGCTTACAACTGTCCTATTCAGTACGCTGTGAGCCCTAAGGCATCTGGCAATAAGC
 393  P  N  Y  A  N  A  Y  N  C  P  I  Q  Y  A  V  S  P  K  A  S  G  N  K  P
1891  CAGACGAAAAGTATACTGGAGAAGTGGTCCCATACCACTGGGAGCACACTGATTATGACTACTTCCAGCC
 417    D  E  K  Y  T  G  E  V  V  P  Y  H  W  E  H  T  D  Y  D  Y  F  Q  P
1961  AAAGATGTTCTGGAAGGTTCTTGGCAGGACGCCAGGCGAACAGGAGTCCCTTGTCAAGAACGTTGCAAAC
 440   K  M  F  W  K  V  L  G  R  T  P  G  E  Q  E  S  L  V  K  N  V  A  N
2031  CATGTTTCTGCTGCTGACGAGTTCATCCAGGACCGTGTTTACGAATACTTCTCGAAGGCCGAGCCTATTA
 463  H  V  S  A  A  D  E  F  I  Q  D  R  V  Y  E  Y  F  S  K  A  E  P  I  I
2101  TTGGAGATCTGATCAGAAAGAAGGTTCAGGAGCTCAAAAGAAAGGCTTCTTCTCCATCCAAAATATAATC
 487    G  D  L  I  R  K  K  V  Q  E  L  K  R  K  A  S  S  P  S  K  I  *
2171  ATCGATAAGTATTTATTGAATCAGTGTTCGTATACTATATTTTTTTTCATTATCTGCCTGATTTTTTTTT
2242  CCCTGATTATAGCTTCCTGATCTGGCAGTCCTGTCCGCCGTATCATCCTTCCTAAAACAGCACGAGATAT
2311  CGGTATATTATAAGAAGCGTATTTTTCCAATCATTATCTTTATTTTTCATCGTTCATGGCTTGTGGATGT
2381  TCCGGTAACAAGAGGACGCAGACCGTCACCGTCCAGCGTCCGCAGAATCTCGTTTCTGAAAAGTCGTCGA
2451  AGCCATCGACCTGCTGTGGCGGCAAGGAGGAAACCAGGTGCAAGTGCGGATCTGCTTGCAAGTGTCCAAG
2521  TTGTAACACCACCAAGTAAGTATCAACTTATTCATCATGCTAACTCCCAGACTGTGAAGAGCTTGTCCGG
2591  ATGTGCTCAAATCATACAGAAGAAGAGATC
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Fig. 3. Nucleotide sequence and deduced amino acid sequence of the catalase gene from *H. polymorpha*. The sequences have been submitted to the EMBL Data Library and given the accession number X 56501.

gradient in contrast to control lysates of pCAT-B transformants, where more than 30% of the total catalase activity was found at a lower position of the gradient (Fig. 4).

The presented evidence for a C-terminal signal of catalase from the methylotrophic yeast *H. polymorpha* for sorting and translocation across the peroxisomal membrane is the first hint at such a function within this universal peroxisomal protein. The data are in agreement with C-terminal tripeptides being targeting signals in peroxisomes as, for example, the common S-K-L-COOH motif originally discovered in the firefly luci-

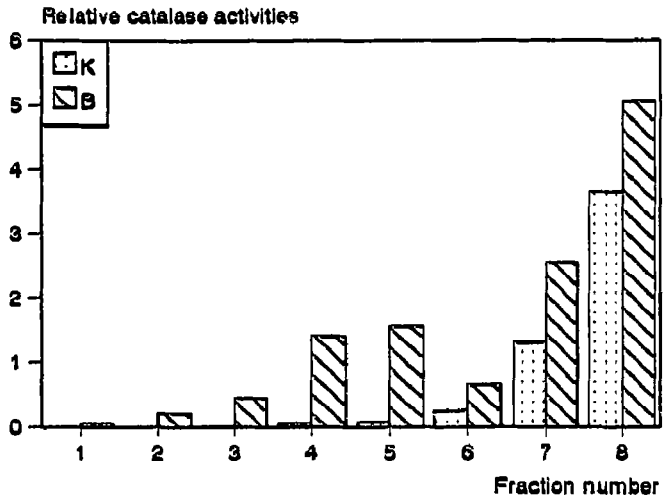

Fig. 4. Sucrose gradient centrifugation of cell lysates from pCAT-K transformants. Experimental conditions were as described in [15]. Increasing fraction numbers of the linear gradient (30–60% sucrose) are from the bottom to the top. K, INT-13/pCAT-K; B, INT-13/pCAT-B.

ferase and rat liver acyl-CoA oxidase [3,4]. This sequence was shown, by expression studies in mammalian cells, to be degenerated to a certain extent as indicated by the consensus sequence S-,A-,C-,/K-,H-,R-,/L [3]. Therefore, the S-K-I signal most probably represents a novel type of sequence, suggesting a wider variation of tripeptide signals, as originally proposed [3]. This view is supported by studies on the *Candida tropicalis* trifunctional enzyme that identified the sequence A-K-I-COOH to be essential for targeting [19]. Thus, an isoleucine at the third position may have developed in yeast.

Recently, an S-K-I-COOH motif was also found in epoxide hydrolase of rodent liver [20]. Since this enzyme is localized in both peroxisomes and the cytoplasm, the authors concluded that S-K-I may be an inefficient target signal in mammalian cells and therefore lead to the bicompartmental distribution.

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