

Two Independent Peroxisomal Targeting Signals in Catalase A of *Saccharomyces cerevisiae*

Fritz Kragler, Alfred Langeder, Jana Raupachova, Maximilian Binder,* and Andreas Hartig

Institut für Biochemie und Molekulare Zellbiologie der Universität Wien and Ludwig Boltzmann-Forschungsstelle für Biochemie, Vienna Biocenter, A-1030 Wien, Austria; *Institut für Tumorbioogie-Krebsforschung der Universität Wien, A-1090 Wien, Austria

Abstract. In contrast to many other peroxisomal proteins catalase A contains at least two peroxisomal targeting signals each sufficient to direct reporter proteins to peroxisomes. One of them resides at the extreme carboxy terminus constituting a new variant of this signal, -SSNSKF, not active in monkey kidney cells (Gould, S. J., G. A. Keller, N. Hosken, J. Wilkinson, and S. Subramani 1989. *J. Cell Biol.* 108:1657-1664). However, this signal is completely dispensable for import of catalase A itself. In its amino-terminal third this protein contains another

peroxisomal targeting signal sufficient to direct reporter proteins into microbodies. This internal signal depends on the context. The nature of this targeting signal might be a short defined sequence or a structural feature recognized by import factors. In addition, we have demonstrated that the carboxy-terminal seven amino acids of citrate synthase of *Saccharomyces cerevisiae* encoded by CIT2 and containing the canonical -SKL represents a targeting signal sufficient to direct reporter proteins to peroxisomes.

IMPORT of proteins into various organelles of the eukaryotic cell is thought to require defined targeting signals. These signals basically direct any protein into the target organelle. Thus far only the nuclear localization signal is positioned in the internal part of an amino acid sequence (Silver, 1991). All other targeting signals found are located either at the amino terminus or at the carboxy terminus (Verner and Schatz, 1988). Most probably, proteins destined for import into an organelle are recognized by chaperone-like proteins keeping the nascent polypeptide chain in an import-competent state (Deshaies et al., 1988; Landry and Gierasch, 1991). The sequence or the molecular structure recognized by chaperones is not known (Gething and Sambrook, 1992). In any case, the partially folded polypeptides finally interact with specific receptors followed by incorporation into the matrix or the membrane of the target organelle, e.g., the mitochondrion (Neupert et al., 1990).

Proteins found in microbodies are usually synthesized at their mature size (Borst, 1989) and the peroxisomal targeting signal is thought to be a short tripeptide at the extreme carboxy terminus (Gould et al., 1987, 1989, 1990) consisting of serine-lysine-leucine or a similar amino acid sequence. However, some peroxisomal proteins do not contain such a sequence at their carboxy termini (see Gould et al., 1989). Examples are peroxisomal catalases (Schroeder et al., 1982; Korneluk et al., 1984; Redinbaugh et al., 1988; Cohen et al., 1988) and peroxisomal 3-ketoacyl-CoA thiolases (Bout et al., 1988; Bodnar and Rachubinski, 1990; Hijikata et al., 1990) from various organisms. For the two peroxisomal thio-

lases from rat, which are synthesized as precursors with amino-terminal presequences of 36 and 26 amino acids, respectively, the amino termini comprise the peroxisomal targeting signal (Swinkels et al., 1991). They are necessary for targeting the thiolases into peroxisomes and sufficient to direct otherwise cytosolic proteins into peroxisomes.

Catalases are among the best-characterized peroxisomal proteins and three-dimensional structures of some of them are known (Murthy et al., 1981; Fita and Rossmann, 1985; Vainshtein et al., 1986). All eukaryotic peroxisomal catalases are tetrameric hemoproteins assembled inside the organelle (for review see Lazarow and Fujiki, 1985). Catalases are devoid of the conserved tripeptidyl peroxisomal targeting signal at their carboxy termini, although some contain fairly similar sequences in this position like -SKF (e.g., Cohen et al., 1988; Orr et al., 1990), which has been shown to be inactive in monkey kidney cells (Gould et al., 1989). Here we describe experiments to locate a peroxisomal targeting signal in the catalase A of *Saccharomyces cerevisiae*.

Materials and Methods

Yeast Strains

The yeast strains GA1-8C (a leu2 ura3 his3 trp1 ctt1 gal1; Simon et al., 1991) and GCI-8B (a leu2 trp1 ura3 ctt1 ctal; Cohen et al., 1985) were used throughout this study. The former one was used for experiments with fusion proteins, the latter one for experiments with catalase A and its variants. Yeast was transformed by the Lithium acetate method (Ito et al., 1983).

Growth Conditions

Transformants of yeast were cultured in synthetic media containing 0.67% Yeast Nitrogen Base without amino acids (Difco Laboratories, Inc., Detroit, MI), 0.5% glucose, and an appropriate mixture of amino acids (S-Cura). After growth at 30°C with shaking until glucose concentration was very low (~0.05%, usually 12 to 16 h) cells were harvested by centrifugation and resuspended in twice the original volume of induction medium containing 30 mM potassium phosphate (pH 6.0), 0.3% yeast extract, 0.5% peptone, 0.2% oleic acid (adjusted to pH 7 with NaOH), and 0.02% Tween 80. After shaking for 16 h at 30°C cells were harvested by centrifugation.

Escherichia coli

The *E. coli* strain HB101 (Bolivar and Backmann, 1979) was used for all transformations and plasmid isolations. For in vitro mutagenesis and M13-phage isolation the strain TGI (Amersham International, Herpenden, UK) was used.

DNA Methods

Isolation of plasmids from *E. coli* was carried out as described (Birnboim and Doly, 1979). Standard procedures were used for cloning and hybridization of DNA (Maniatis et al., 1982). Linear fragments were isolated from the agarose gels as described (Dretzen et al., 1981). Restriction enzymes were obtained from Boehringer-Mannheim GmbH (Mannheim, Germany) and used as recommended. Double strand sequencing was performed using the T7 sequencing kit from Pharmacia (Uppsala, Sweden). Oligonucleotides were kindly supplied by Dr. G. Schaffner (Institute for Molecular Pathology, Vienna, Austria).

Plasmid Construction and Mutagenesis

All plasmids used in yeast are derived from the multicopy plasmid YEp352 (Hill et al., 1986). Gene fusions between different parts of the CTA1 gene and the cDNA encoding dihydrofolate reductase (DHFR)¹ of mouse under the control of the CTA1 promoter cloned into this vector were available from previous work (Hartig et al., 1990). The cDNA encoding DHFR (0.65 kb BamHI-HindIII fragment) was replaced with a BamHI-HindIII fragment coding for subunit IV of cytochrome c oxidase (cox IV; Maarse et al., 1984) of *Saccharomyces cerevisiae* or with modified versions of DNA-sequences

1. *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; PCR, polymerase chain reaction.

encoding one of the two reporter proteins. DNA containing cox IV from *S. cerevisiae* encoding a truncated version of subunit IV without the 20 NH₂-terminal amino acids representing the mitochondrial signal sequence was a kind gift from Dr. A. P. G. M. van Loon (Hoffmann LaRoche AG, Basel, Switzerland). The DNA fragment contained a BamHI-restriction site in the same reading frame as used previously for DHFR and a HindIII site downstream of the stop codon. Modifications in the DNA-sequence of both reporter proteins to alter their carboxy termini were introduced with oligonucleotide-directed mutagenesis carried out on single strand DNA using a kit from Amersham International. Oligonucleotides used for mutagenesis and the corresponding amino acid changes are summarized in Tables I and II. The three different modifications of the cox IV part were introduced with three different oligonucleotides. The cDNA of DHFR was first mutagenized to introduce the sequence -SKL onto the carboxy terminus. In a second mutagenesis the carboxy terminus was modified to resemble the carboxy terminus of citrate synthase or luciferase. The last six codons of CTA1 were fused to the cDNA of DHFR by eliminating 10 codons of CTA1 from a clone constructed earlier (Hartig et al., 1990) containing the last 16 codons of CTA1 at the 3' end of the cDNA of DHFR.

Oligonucleotide-directed mutagenesis on single strand DNA was also applied to eliminate the last three codons of catalase A or to introduce the amino acid sequence Arg-His-His-His (RHHH) instead of the penultimate amino acid lysine (K) or to eliminate the sequence from amino acid 369 to 416. The modified DNA-sequence was used to replace the wild type coding sequence in the vector YEp352 containing the 2.7 kb EcoRI fragment of the complete CTA1 gene (Cohen et al., 1988) but missing the multiple cloning site except the EcoRI recognition site. The same plasmid was used to delete the part of the DNA encoding the sequence between amino acid 3 and 43 or 111 with polymerase chain reaction (PCR; Saiki et al., 1988; Ho et al., 1989), using the unique restriction sites for SacI and BamHI in the primer sequences (lines 2, 3, and 4 in Table II) to replace the corresponding wild type fragments. The same plasmid was also used as template to introduce the deletion between bp 378 and bp 427 of the catalase A coding region (between amino acid 126 and amino acid 143). Two parallel PCRs were carried out using primers depicted in Table II (lines 4 and 5 and lines 6 and 7, respectively). The products were purified by gel electrophoresis, hybridized together and amplified using the "outside primers" (lines 4 and 7 in Table II). The resulting DNA fragment was cut with SacI and BamHI, purified, and used to replace the SacI-BamHI fragment of the wild type gene. Mutagenesis by PCR was also used to introduce deletions into the catalase A part of fusion proteins consisting of a reporter protein and 126 or 140 amino acids of catalase. Primers are shown in lines 2, 3, and 8 in Table II, plasmids containing gene fusions with the cDNA of DHFR were used as templates (Hartig et al., 1990), and the cDNA of

Table I. Oligonucleotides Used for In Vitro Mutagenesis at the Carboxy Termini

Oligonucleotide sequence	Position (gene)	Amino acid sequence	Comments
GATGACCACCATAAAAACATTGAAAGCAAACATAATCTTATCATTC	+452/+479 (coxIV)	-HKNIESKL*	citrate synthase-terminus on subunit IV of cytochrome c oxidase
GATGACCACCATGGCGGAAAGTCCAAATTGTAATCTTATCATTC	+452/+479 (coxIV)	-HGGSKSL*	luciferase-terminus on subunit IV of cytochrome c oxidase
GATGACCACCATTGAGTAACCTCAAATTTAATCTTATCATTC	+452/+479 (coxIV)	-HSSNSKF*	catalase-terminus on subunit IV of cytochrome c oxidase
CCTGTTAAAGTTTACTCTTCTCG	+568/+549 (cDNA of DHFR)	-EKSKL*	carboxy-terminal modification in DHFR
GTTAAAGTTTACTTTCAATGTTTTCTTCTCGTAGAC	+565/+544 (cDNA of DHFR)	-EKKNIESKL*	citrate synthase-terminus on DHFR
GTTAAAGTTTACTCTTCCGCCCTTCTCGTAGAC	+565/+544 (cDNA of DHFR)	-EKGGKSKL*	luciferase-terminus on DHFR
GGAGTTACTCGACTTCTCGTAGAC	+1539/+544 (CTA1/cDNA of DHFR)	-EKSSNSKF*	catalase-terminus on DHFR
CTTAAGCTTGTCGACTCAGTTACTCGA	+1558/+1528 (CTA1)	-SELSSN*	carboxy-terminal modification in catalase
ACTTAAGCTTTTCAAAAATGATGATGACGGGAGTTAC	+1559/+1532 (CTA1)	-NSRHHHF*	carboxy-terminal modification in catalase

All oligonucleotides used for mutagenesis at the carboxy termini are listed with the position of hybridization in the corresponding gene or cDNA and the new amino acid sequence (larger bold print). Ends of amino acid sequences are indicated by asterisks. Amino acid sequences of the carboxy termini of the authentic proteins are given for comparison: catalase A, -SSNSKF (Cohen et al., 1988); DHFR, -VYEKKD (Stone and Phillips, 1977); and subunit IV of cytochrome c oxidase, -NDDHHH (Maarse et al., 1984).

Table II. Oligonucleotides Used for Deletions in CTA1

Oligonucleotide sequence	Position	Amino acids fused	Comments
CCTAACTTCCAT - CCCATTCAACAAC	+1093/+1261	..NFH-PIQ.. (368-417)	deletion between basepair 1104 and 1249
GAGCTCTAGAAGATGTCCAAA - GGCCCTTTGCTTTTGAAGATTAT	-13/+150	MSK-GPL.. (3-43)	deletion between basepair 9 and 127
GGAGCTCTAGAAGATGTCCAAA - GTGGGTGGTGATAAAGGTAGTGC	-13/+353	MSK-VGG.. (3-111)	deletion between basepair 9 and 331
CTGGATTGGATCCCGCAAT	+766/+748		primer for PCR
CGTGATCCAAGGGGG - AATAATACACCGGTAT	+364/+442	..PRG-NNT.. (126-143)	deletion between basepair 378 and 427
CCCCCTTGGATCACG	+378/+364		primer for PCR
TAAATTTGGAGCTCTAGAAGATG	-20/+3		primer for PCR
GACGATGCAGTTC AATGGTCCG	+27/+7		primer for PCR
	(cDNA of DHFR)		

Oligonucleotides used for mutagenesis are listed with their position of hybridization and the resulting amino acid sequence. Deletions are indicated by hyphen. The first oligonucleotide listed was used for mutagenesis of single strand DNA, all others for mutagenesis with the PCR.

DHFR was replaced by the truncated *coxIV* gene as described above using the unique restriction sites BamHI and HindIII.

Preparation of Organellar Pellet and Immunoblot Analysis

Yeast cells were harvested by centrifugation (~250 ml cell suspension, 2×10^7 cells/ml), washed once with water, and converted to spheroplasts by incubation in 0.1 M Tris-SO₄ (pH 9.4), 10 mM DTT for 10 min at 30°C and in 1.2 M sorbitol, 20 mM potassium phosphate (pH 7.4) containing 5 mg/g cells zymolyase 20T (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) for 30 to 60 min at 30°C. After centrifugation at low speed these spheroplasts were resuspended in ice-cold 20 ml of 0.6 M sorbitol, 5 mM MOPS (pH 6.0), 1 mM KCl, 0.5 mM EDTA, 1 mM PMSF, and homogenized by 12 strokes with a Dounce homogenizer (Kontes Glass, Vineland, NJ) (1,000 rpm). The pellet after a short centrifugation at 2,000 g was homogenized again by six strokes with the homogenizer, and after centrifugation at 2,000 g the supernatant was united with the supernatant of the first centrifugation. For separation of organelles from the remaining cytosol the supernatants were centrifuged at 25,000 g for 15 min. The resultant fractions are referred to as organellar pellet and postorganellar supernatant. Protein was determined with the method of Bradford (Bradford, 1976). Equal amounts of protein were separated by SDS-PAGE (Laemmli, 1970) and blotted onto nitrocellulose as described (Burnette, 1981).

Antibodies

Antibodies against catalase A and against DHFR were from goat. Rabbit antibodies against subunit IV of cytochrome c oxidase were a kind gift from Dr. G. Schatz (University of Basel, Basel, Switzerland). For detection of the respective proteins on Western-blots the first antibodies were diluted 1:1,000 in 5% wt/vol non-fat dry milk in TBS. The second antibodies, either alkaline phosphatase-conjugated anti-goat antibodies from rabbit or alkaline phosphatase-conjugated goat anti-rabbit antibodies (Axell, Westbury, NY), were used in a dilution of 1:2,500 in 5% wt/vol non-fat dry milk in TBS and visualized with a combination of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitro blue tetrazolium chloride (Robinson et al., 1988).

EM

Yeast cells were harvested from the induction medium containing oleic acid by centrifugation (50 ml cell suspension, $\sim 4 \times 10^7$ cells/ml). After two washes in 5 ml medium without carbon source, the cells were resuspended in 2.5 ml of the same medium. An equal volume of ice-cold 2× fixative (8% formaldehyde, freshly prepared from paraformaldehyde, and 4% glutaraldehyde in 2× PBS (80 mM K₂HPO₄, 20 mM KH₂PO₄, 300 mM NaCl, 0.2% NaN₃, pH 7.3) was added rapidly to ensure uniform mixing. After incubation for 5 min at room temperature the cells were harvested by centrifugation and resuspended in 1× fixative (5 ml). Fixation continued on ice for 30 min. Subsequently, cells were washed three times in PBS. Cell wall carbohydrates were oxidized by incubation in 1% sodium metaperio-

date followed by ammonium chloride treatment as described (Van Tuinen and Riezman, 1987). Thereafter, several rinses with PBS were followed by inclusion of cells in low-melt agarose. Low temperature dehydration and embedding in Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, Germany) was carried out as described (Carlemalm et al., 1982). After polymerization, 90-nm thick sections were cut with a diamond knife and mounted on carbon-parlodion-coated nickel grids (300 mesh). Immunocytochemical staining was performed essentially as described (Roth et al., 1978). For blocking of unspecific binding sites, grids were floated on drops of PBS containing 1% BSA for 5 min at room temperature. After blocking, the grids were placed on drops of antibody solution (diluted in PBS containing 1% BSA, 0.1% Triton X-100, 0.1% Tween 20, and 0.02% NaN₃) and incubated for 2 h at room temperature. Three washes in PBS were followed by incubation with protein A-gold (diluted to an OD of 0.44 at 525 nm with PBS containing BSA, Triton 20, and NaN₃ as above) for 1 h at room temperature. Protein A-gold complexes were prepared as described earlier (Binder et al., 1986). Finally, the grids were washed twice in PBS and once in distilled water. Air-dried sections were poststained with 2% uranyl acetate and Millonig's lead acetate (Millonig, 1961). Sections were viewed in a Philips electron microscope (Philips, Eindhoven, The Netherlands). For control of the specificity of the labeling procedures for immunoelectron microscopy, the respective primary antibody in the incubation mixture was replaced by non-immune serum.

For each construct at least 10 sections equalling ~700 peroxisomal profiles were looked at. We considered the frequency of occurrence of labeled peroxisomes as: (a) negative (-), if none of the inspected peroxisomal profiles were labeled; (b) borderline (+/-), if up to 1% of inspected peroxisomal profiles were labeled; and (c) positive (+), if more than 1% of inspected peroxisomal profiles were labeled.

Results

Construction of Gene Fusions and Deletions in the CTA1 Gene

Many fusion proteins consisting of the entire DHFR at the carboxy terminus and different parts of the 515 amino acids of catalase A at the amino terminus did not enter peroxisomes of *S. cerevisiae* (Hartig et al., 1990). Therefore we decided to test the influence of the reporter protein and of the carboxy termini of different peroxisomal proteins (luciferase from firefly [-GGKSKL], citrate synthase from *S. cerevisiae* encoded by CIT2 [-KNIESKSL], and catalase A [-SSNSKF]) on import into peroxisomes. A summary of the constructs and of the resulting products is shown in Figs. 1 and 2.

Modifications in the coding region of CTA1 gave rise to catalase proteins with altered carboxy termini or with inter-

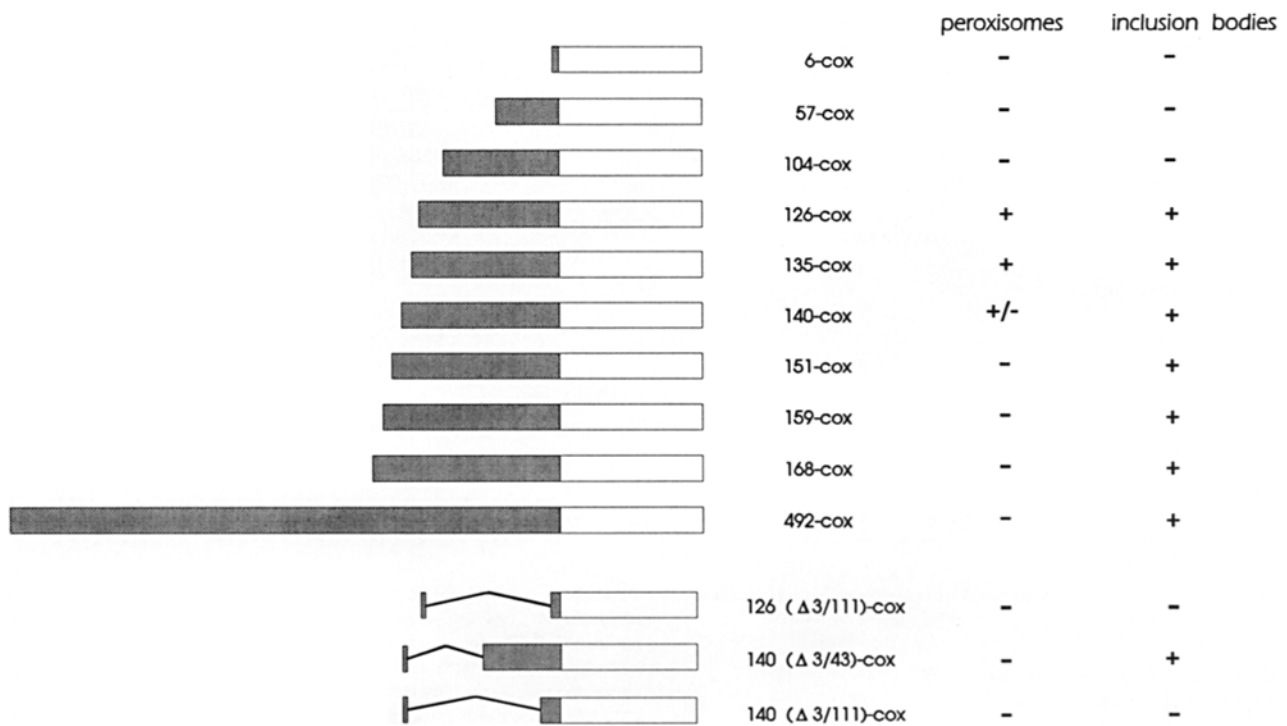


Figure 1. Intracellular location of fusion proteins. The intracellular location of fusion proteins consisting of a variable number of amino acids of catalase A at the amino terminus and the reporter protein subunit IV of cytochrome c oxidase was determined by immunoelectron microscopy. The numbers symbolize the length of the amino acid sequence from catalase A at the amino terminus. Deletions in the catalase part of fusion proteins are indicated in brackets with the last amino acid of the sequence still present and the first amino acid already present in the hybrid protein. The cytoplasmic location of some of the proteins led to proteinaceous aggregates (inclusion bodies) due to over-expression.

nal deletions. These include proteins missing the last three amino acids of catalase A or those whose penultimate amino acid lysine (K) was replaced with the sequence Arg-His-His (RHHH) as well as proteins missing sequences from amino acid 4 to 42 or from 4 to 110 or from 127 to 142 or

from amino acid 369 to 416. In addition, fusion proteins consisting of the entire sequences of catalase A and DHFR have been constructed by appropriate gene fusions. A summary of the catalase variants is shown in Fig. 4.

Relative amount of all proteins analyzed was determined.

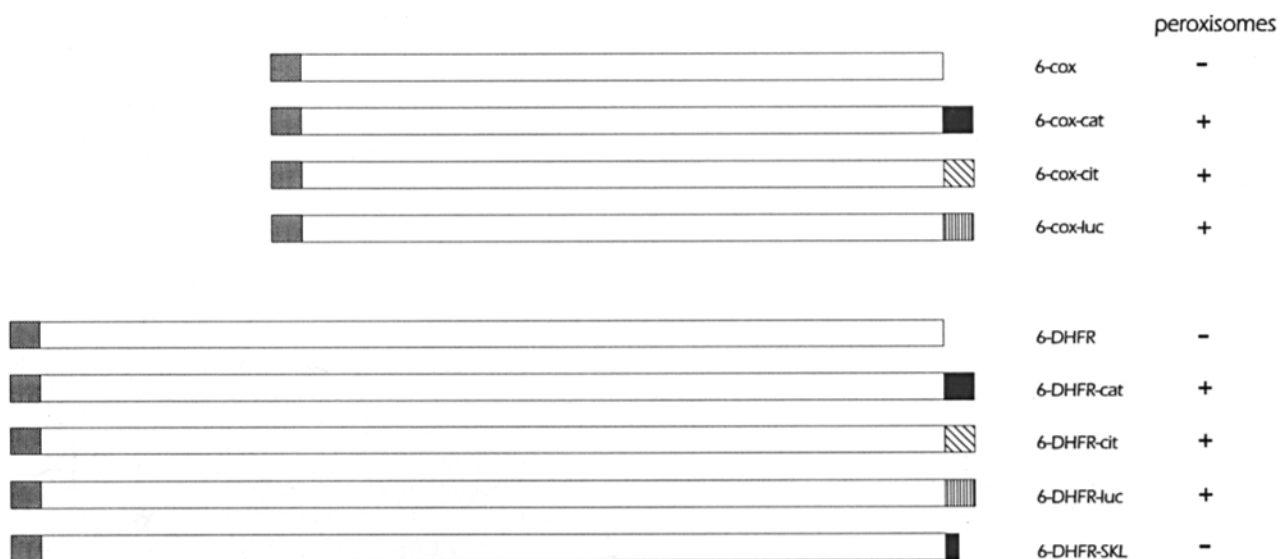


Figure 2. Intracellular location of fusion proteins. The intracellular location of fusion proteins consisting of the six amino-terminal amino acids from catalase A at the amino terminus, a reporter protein (DHFR or subunit IV of cytochrome c oxidase) and different carboxy termini was determined by immunoelectron microscopy. The letters indicate the six carboxy-terminal amino acids from catalase A (*cat*) or from luciferase (*luc*) or the seven carboxy-terminal amino acids from citrate synthase (*cit*) at the carboxy terminus of the fusion protein, or an artificial carboxy terminus consisting of Serine-Lysine-Leucine (SKL).

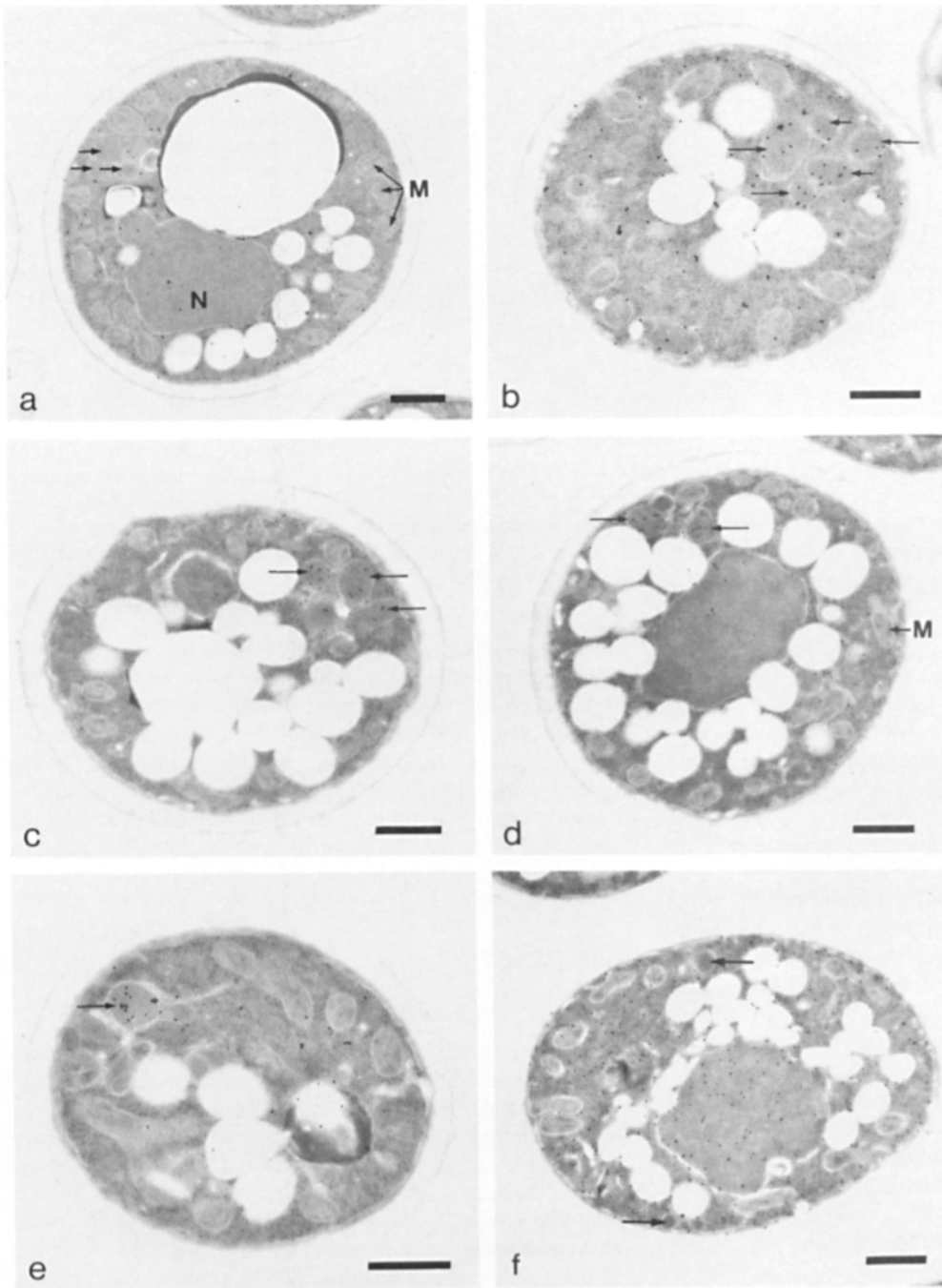


Figure 3. Localization of hybrid proteins with the electron microscope by immunogold staining. Yeast cells (strain GA1-8C) transformed with plasmids containing different gene fusions were prepared for electron microscopy as described (see Materials and Methods). Thin sections were reacted with anti-cytochrome c oxidase antibody and protein A-gold (14 nm, dilution 1:60). Abbreviations used for gene fusions are the same as in Figs. 1 and 2. *N*, nuclei; *M*, mitochondria; *I*, inclusion body. (a) 57-cox. Antibody diluted 1:9,000. Nucleus and cytoplasm are sparsely labeled. No label over peroxisomes (arrows). (b) 6-cox-cat. Antibody diluted 1:6,000. Note labeled peroxisomes (arrows). (c) 6-cox-luc. Antibody diluted 1:9,000. Note labeled peroxisomes (arrows). (d) 126-cox. Antibody diluted 1:9,000. Peroxisomes are labeled (arrows). Few gold particles are seen over the nuclear profile. (e) 140-cox. Antibody diluted 1:3,000. Note labeled peroxisome (arrow). (f) 126 ($\Delta 3/111$)-cox. Antibody diluted 1:6,000. Note unlabeled peroxisomes (arrows) and label over nucleus and cytoplasm. Bars: 0.5 μ m.

Fusion proteins and all catalase A variants were immunologically detected on Western blots (Burnette, 1981) of proteins from crude organellar pellets and from postorganellar supernatants (results not shown). Basically, no major differences in expression levels and stability were found.

Intracellular Location of Fusion Proteins

The intracellular location of fusion proteins was determined with the electron microscope only, since biochemical techniques might be misleading due to formation of inclusion bodies (Hartig et al., 1990). Representative electron micrographs are shown in Fig. 3, results are summarized in Figs. 1 and 2. Hybrid proteins with small parts of catalase A (e.g., 6-cox, 57-cox, or 104-cox) were not imported into peroxi-

somes (Figs. 1 and 3 a). However, the fusion protein consisting of 126 amino acids of catalase A at the amino terminus and subunit IV of cytochrome c oxidase was imported into peroxisomes (Fig. 1). Nearly each peroxisome was labeled with antibodies against subunit IV of cytochrome c oxidase (Fig. 3 d). Two slightly larger fusion proteins (135 or 140 amino acids of catalase A and subunit IV of cytochrome c oxidase) were found in the peroxisomal compartment, too (see Figs. 1 and 3 e). However, frequency of labeled peroxisomes was much lower. Larger fusion proteins (151, 159, 168, or 492 amino acids and subunit IV of cytochrome c oxidase) did not enter peroxisomes at all. They were usually located in the cytoplasm, often aggregated to large inclusion bodies. This resembles the situation with catalase A itself,

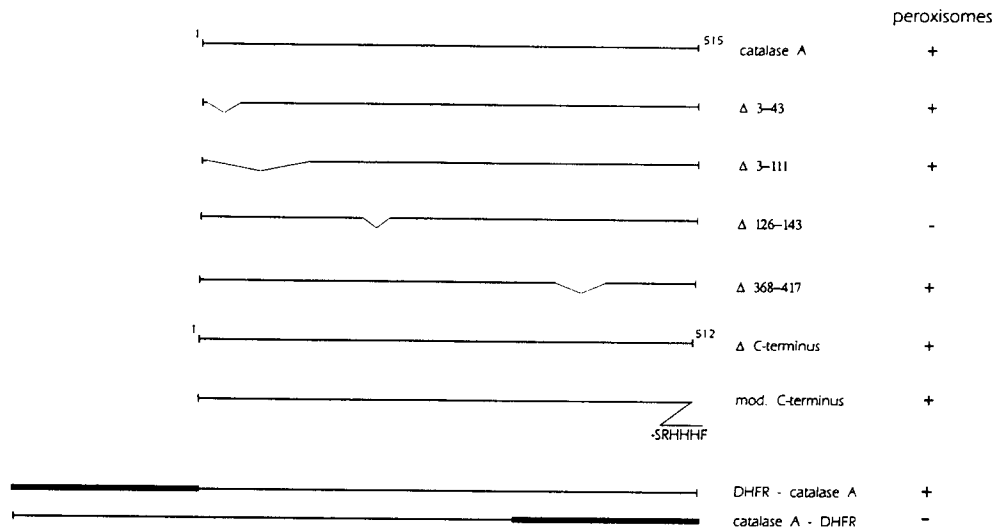


Figure 4. Intracellular location of catalase A and variants. The intracellular location of catalase A and variants thereof was determined by immunoelectron microscopy. The carboxy termini of the proteins consisted of the authentic carboxy terminus of catalase A (-SSNSKF, line 1-5 and 8) except for the hybrid protein catalase A-DHFR (line 9) and where indicated (lines 6 and 7). Catalase A was modified at the carboxy terminus by deletion of the last three amino acids serine-leucine-leucine (SKF, line 6) or by insertion of arginine-histidine-histidine in-

stead of lysine (-SRHHHF, line 7). The deletions are indicated with numbers representing the last amino acid still present and the first amino acid already present in the new protein. Due to overexpression the cytoplasmic location of the proteins led in some cells to proteinaceous aggregates (inclusion bodies), whose appearance is not indicated in this figure.

which upon overexpression is also found aggregated in the cytoplasm besides its peroxisomal location (Binder et al., 1991). Aggregation and import into peroxisomes are not mutually exclusive, as was demonstrated for catalase A itself (Binder et al., 1991) and some fusion proteins in this work. Deletion of parts of the catalase A sequences from fusion proteins found inside peroxisomes (e.g., 126-cox and 140-cox) resulted in the loss of import competence (Figs. 1 and 3 f).

Slight modifications at the carboxy termini of import incompetent hybrid proteins (e.g., 6-DHFR or 6-cox) by addition of few amino acids resembling the carboxy termini of peroxisomal proteins allowed import into peroxisomes (see Fig. 2). These carboxy termini consisted of the six carboxy-terminal amino acids from luciferase (de Wet et al., 1987), or of the seven carboxy-terminal amino acids of the peroxisomal citrate synthase (Rosenkrantz et al., 1986; Lewin et al., 1990) or of the six carboxy-terminal amino acids of catalase A (Cohen et al., 1988). Examples are depicted in Fig. 3, b and c. Addition of the signal tripeptide SKL (Gould et al., 1988) to the carboxy terminus of the fusion protein 6-DHFR was not sufficient for a peroxisomal location of the resultant polypeptide (see Fig. 2).

Intracellular Location of Modified Catalase A Proteins

Results with the modified catalase A proteins are summarized in Fig. 4 and examples are depicted in Fig. 5. In contrast to the results described above indicating a signalling function at the carboxy terminus of catalase A the deletion of the last three amino acids (SKF) of catalase A did not alter the peroxisomal location of the protein. Similarly, a catalase protein whose penultimate amino acid lysine (K) was replaced by the tetrapeptide Arg-His-His-His (RHHH) was imported into peroxisomes. Deletions in the more amino-terminal part of catalase A (deletion 3-111, deletion 3-43) had little or no effect on import, whereas a protein devoid of the sequence between amino acid 126 and 143 could not be found inside peroxisomes. Deletion of the region consisting of the sequence between amino acid 368 to amino acid

417 had no effect on import. This region was originally proposed as a possible candidate for a targeting signal, since the differences to the cytoplasmic catalase T are pronounced (Cohen et al., 1988). Major modifications by adding the 187 amino acid sequence encoding DHFR from mouse to the carboxy terminus of catalase A resulted in a cytoplasmic location of the hybrid protein. However, a fusion protein of the same size with catalase A at the carboxy terminus was imported into peroxisomes.

Discussion

Carboxy-terminal Peroxisomal Targeting Signal

In Fig. 2 results are summarized indicating that carboxy termini of three different proteins are sufficient for the peroxisomal location of hybrid proteins. Although citrate synthase encoded by the CIT2 gene was shown to be imported into peroxisomes of *S. cerevisiae* (Lewin et al., 1990) and although the carboxy terminus of this protein consists of SKL (Rosenkrantz et al., 1986), it was the experiment described above that identified a peroxisomal targeting signal residing in the seven carboxy-terminal amino acids of this protein. The carboxy terminus of catalase A consists of the amino acid sequence -SKF, which is not a peroxisomal targeting signal for luciferase in monkey kidney cells (Gould et al., 1989). Nevertheless, the six carboxy-terminal amino acids of catalase A were sufficient to direct proteins into peroxisomes of *S. cerevisiae*. Most published experiments are similarly designed, and the conclusions drawn from these results are certainly correct: the carboxy termini of various peroxisomal proteins are sufficient to direct otherwise cytosolic proteins into peroxisomes. Variants of the carboxy-terminal signal are tolerated in lower eukaryotic organisms as indicated by signals like -AKI in the trifunctional enzyme of *Candida tropicalis* (Aitchinson et al., 1991) or -SKI, -NKL, and -ARF for catalase (Didion and Roggenkamp, 1992), dihydroxyacetone synthase, and methanol oxidase in *Hansenula polymorpha*, respectively (Hansen et al., 1992).

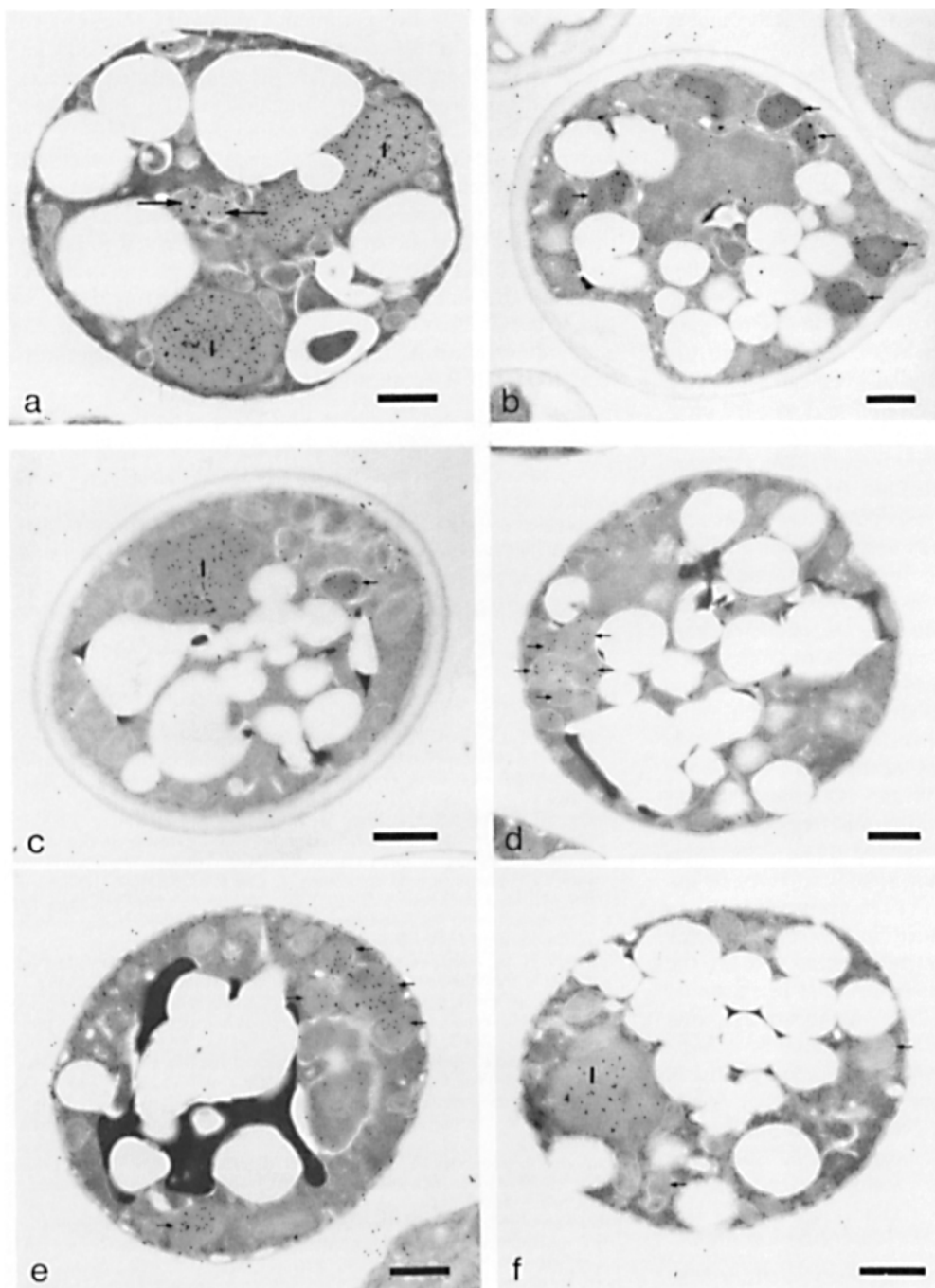


Figure 5. Localization of catalase A and modified catalase-proteins with the electron microscope by immunogold staining. Yeast cells (strain GC1-8B) transformed with multicopy plasmids harboring the wild type gene or modified versions thereof were prepared for EM as described (see Materials and Methods). Thin sections were reacted with anti-catalase antibody and subsequently with protein A-gold (14 nm, dilution 1:60). *N*, nuclei; *M*, mitochondria; *I*, inclusion body. (a) Wild-type catalase A with modified carboxy terminus (-SRHHHF instead of SKF) encoded by the plasmid. Antibody diluted 1:200. Note labeled peroxisomes (arrows) and labeled inclusion bodies (*I*). (b) Wild type CTA1 gene on the plasmid. Anti-catalase antibody diluted 1:400. Peroxisomes are labeled (arrows). (c) Wild-type catalase A with modified carboxy terminus (without SKF) encoded by the plasmid. Antibody diluted 1:400. Note labeled inclusion body (*I*) and labeled peroxisome (arrow). (d) Catalase A devoid of the sequence between amino acid 3 and 43 (Δ 3-43) encoded by the plasmid. Antibody diluted 1:300. Note labeled peroxisomes (arrows). (e) Catalase A devoid of the sequence between amino acid 368 and 417 (Δ 368-417) encoded by the plasmid. Antibody diluted 1:400. Note labeled peroxisomes (arrows). (f) Catalase A devoid of the sequence between amino acid 126 and 143 (Δ 126-143) encoded by the plasmid. Antibody diluted 1:500. Note labeled inclusion body (*I*). Peroxisomes are not labeled (arrows). Bars: 0.5 μ m.

In most cases the carboxy termini are also necessary for import into the organelle, e.g., of luciferase (Gould et al., 1989). However, deletion of the authentic carboxy terminus of catalase A or major modifications in the carboxy terminus did not change the peroxisomal location of catalase A (see Fig. 4). The only possible conclusion from these results is that catalase A possesses at least one other peroxisomal targeting signal. This signal(s) must reside somewhere in the amino acid sequence or in structural features of the import-competent protein.

Location of the Internal Peroxisomal Localization Signal

Besides the well-established peroxisomal targeting signals at the carboxy terminus (Gould et al., 1987) a peroxisomal localization signal can also be found at an amino-terminal location (Swinkels et al., 1991). Our findings demonstrate the internal location of a peroxisomal localization signal. In Fig. 1 results are summarized indicating a possible peroxisomal localization signal in the first third of the amino acid

sequence of catalase A. 126 to 140 amino-terminal amino acids of catalase A permit the import of a fusion protein with subunit IV of cytochrome c oxidase. With DHFR as reporter protein similar results could be observed with a much lower frequency of labeled peroxisomes (M. Binder, unpublished observation) indicating that DHFR might not be an ideal reporter protein for peroxisomal import studies in *S. cerevisiae*. In addition, this low import seems to be strain dependent, since hybrid proteins with DHFR could not be detected inside peroxisomes of strain A777 used previously (Hartig et al., 1990). The first 104 amino acids of catalase A were not sufficient for import of fusion proteins. If we assume that the reporter protein does not provide any part of the localization signal, the putative peroxisomal targeting signal must reside somewhere in the sequence between amino acid 104 and 126. Comparison in this region between catalases from different eukaryotic organisms and catalase A showed identity of amino acid sequence between 82 and 56% (data not shown) with catalase from *H. polymorpha* (Didion and Roggenkamp, 1992) and maize encoded by cat1 (Redinbaugh et al., 1988) at the extreme ends, respectively. However, the effectiveness of this signal depends very much on the context, since fusion proteins with more than 140 amino acids of catalase A could not be found inside peroxisomes. Additionally, fusion proteins containing only the sequence from amino acid 43 (or 111) to 140 of catalase A or from amino acid 111 to 126 of catalase A besides the first three amino acids of this protein at the amino terminus were not found inside peroxisomes (see Fig. 1). Therefore, it seems too early to confine the internal peroxisomal localization signal to the sequence between amino acid 104 and 126 of catalase A, since we cannot exclude contributions of parts of catalase A or of the reporter protein to the targeting signal. Moreover, only the deletion of a region immediately adjacent to the putative targeting signal, deletion of the sequence between amino acids 126 and 143, resulted in incompetence for peroxisomal import of the respective protein. Already the first paper published on the carboxy-terminal peroxisomal targeting signal (Gould et al., 1987) demonstrated, that somewhere in the amino-terminal half of luciferase a large sequence or structure is present whose alteration abolishes import into peroxisomes. The major difference to our results with catalase A is that for the peroxisomal import of luciferase the carboxy terminus of this protein is indispensable (Gould et al., 1989), whereas for catalase A it is not.

Nature of the Internal Peroxisomal Localization Signal

Many different proteins containing the newly identified internal peroxisomal localization signal were not imported into the organelle (see Figs. 1 and 4). A possible interpretation would be that the recognition of a defined sequence requires exposure of such a signal to the interacting proteins. Therefore, internal signal sequences may depend on the context for exposure to interacting proteins, similar to the nuclear localization sequences (Silver, 1991). Formation of a structure allowing interaction of a targeting sequence would be an intrinsic feature of authentic peroxisomal proteins like catalase A. If the targeting sequence is properly exposed interaction occurs followed by import into peroxisomes.

However, another mode of action could be imagined: the structural features alone and not a distinct contiguous sequence represent the signal and are recognized by specific

cytosolic factors delivering their substrates to peroxisomes. In this case the formation of the "targeting structures" represents already a commitment for import into microbodies. This commitment can be so strong, that the carboxy terminus is not required any more, as demonstrated for catalase A (Fig. 4) and fusion proteins with 126, 135, or 140 amino acids of catalase A at the amino terminus (Fig. 1). The formation and the effectiveness of these "targeting structures" certainly depend on other influences like exposure on the surface, size or overall structure of the protein. Only fusion proteins and catalase variants would be targeted to peroxisomes, in which the formation of this "targeting structure" is permitted. An independent alternative targeting signal exists at the carboxy terminus, which is dispensable in the authentic catalase A, but might be essential in fusion proteins like DHFR-catalase (Fig. 4).

The authors thank R. Krumpfuss and W. Stadler for technical assistance and Dr. M. Simon for fruitful discussions and critical reading of the manuscript.

Financial support by the Ludwig Boltzmann-Gesellschaft and by grant S5810 of the Fonds zur Förderung der wissenschaftlichen Forschung to A. Hartig is gratefully acknowledged.

Received for publication 6 August 1992 and in revised form 27 October 1992.

References

- Aitchinson, J. D., W. W. Murray, and R. A. Rachubinski. 1991. The carboxyl-terminal tripeptide Ala-Lys-Ile is essential for targeting *Candida tropicalis* trifunctional enzyme to yeast peroxisomes. *J. Biol. Chem.* 266:23197-23203.
- Binder, M., S. Tourmente, J. Roth, M. Renaud, and W. J. Gehring. 1986. In situ hybridization at the electron microscope level: localization of transcripts on ultrathin sections of Lowicryl K4M-embedded tissue using biotinylated probes and protein A-gold complexes. *J. Cell Biol.* 102:1646-1653.
- Binder, M., M. Schanz, and A. Hartig. 1991. Vector-mediated overexpression of catalase A in the yeast *Saccharomyces cerevisiae* induces inclusion body formation. *Eur. J. Cell Biol.* 54:305-312.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
- Bodnar, A. G., and R. A. Rachubinski. 1990. Cloning and sequence determination of cDNA encoding a second rat liver peroxisomal 3-ketoacyl-CoA thiolase. *Gene.* 91:193-199.
- Bolivar, F., and K. Backmann. 1979. Plasmids of *Escherichia coli* as cloning vectors. In *Methods in Enzymology*. Vol. 68. Colowick, S. P., and N. O. Kaplan, editors. Academic Press, New York. 245-267.
- Borst, P. 1989. Peroxisome biogenesis revisited. *Biochim. Biophys. Acta.* 1008:1-13.
- Bout, A., Y. Teunissen, T. Hashimoto, R. Benne, and J. M. Tager. 1988. Nucleotide sequence of human peroxisomal 3-oxoacyl-CoA thiolase. *Nucleic Acids Res.* 16:10369.
- Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
- Carlemalm, E., R. M. Garavito, and W. Villiger. 1982. Resin development for electron microscopy and an analysis of embedding at low temperature. *J. Microsc. (Oxf.)* 126:123-143.
- Cohen, G., F. Fessl, A. Traczyk, J. Rytka, and H. Ruis. 1985. Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the cta1 mutation. *Mol. Gen. Genet.* 200:74-79.
- Cohen, G., W. Rapatz, and H. Ruis. 1988. Sequence of the *Saccharomyces cerevisiae* CTA1 gene and amino acid sequence derived from it. *Eur. J. Biochem.* 176:159-163.
- Deshais, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.)* 332:800-805.
- de Wet, J. R., K. W. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725-737.

- Didion, T., and R. Roggenkamp. 1992. Targeting signal of the peroxisomal catalase in the methylotrophic yeast *Hansenula polymorpha*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 303:113-116.
- Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon. 1981. A reliable method for recovery of DNA fragments from agarose and acrylamide gels. *Anal. Biochem.* 112:295-298.
- Fita, I., and M. G. Rossmann. 1985. The active center of catalase. *J. Mol. Biol.* 185:21-37.
- Gething, M. J., and J. Sambrook. 1992. Protein folding in the cell. *Nature (Lond.)* 355:33-45.
- Gould, S. J., G. A. Keller, and S. Subramani. 1987. Identification of a targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.* 105:2923-2931.
- Gould, S. J., G. A. Keller, and S. Subramani. 1988. Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell Biol.* 107:897-905.
- Gould, S. J., G. A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657-1664.
- Gould, S. J., G. A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. F. Tabak, and S. Subramani. 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:85-90.
- Hansen, H., T. Didion, A. Thiemann, M. Veenhuis, and R. Roggenkamp. 1992. Targeting sequences of the two major peroxisomal proteins in the methylotrophic yeast *Hansenula polymorpha*. *Mol. Gen. Genet.* 235:269-278.
- Hartig, A., M. Ogris, G. Cohen, and M. Binder. 1990. Fate of highly expressed proteins destined to peroxisomes in *Saccharomyces cerevisiae*. *Curr. Genet.* 18:23-27.
- Hijikata, M., J. K. Wen, T. Osumi, and T. Hashimoto. 1990. Rat peroxisomal 3-ketoacyl-CoA thiolase gene. Occurrence of two closely related but differentially regulated genes. *J. Biol. Chem.* 265:4600-4606.
- Hill, J. E., and A. M. Myers, T. J. Koerner, and A. Tzagaloff. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast.* 2:163-167.
- Ho, S. N., H. D. Hunt, R. M. Horten, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 77:51-59.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163-168.
- Korneluk, R. G., F. Quan, W. H. Lewis, K. S. Guise, H. F. Willard, M. T. Holmes, and R. A. Gravel. 1984. Isolation of human fibroblast catalase cDNA clones. Sequence of clones derived from spliced and unspliced mRNA. *J. Biol. Chem.* 259:13819-13823.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Landry, S. J., and L. M. Gierasch. 1991. Recognition of nascent polypeptides for targeting and folding. *Trends Biochem. Sci.* 16:159-163.
- Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489-530.
- Lewin, A. S., V. Hines, and G. M. Small. 1990. Citrate synthase encoded by the CIT2 gene of *Saccharomyces cerevisiae* is peroxisomal. *Mol. Cell. Biol.* 10:1399-1405.
- Maarse, A. C., A. P. G. M. Van Loon, H. Riezman, I. Gregor, G. Schatz, and L. A. Grivell. 1984. Subunit IV of yeast cytochrome c oxidase: cloning and nucleotide sequencing of the gene and partial amino acid sequencing of the mature protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2831-2837.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
- Millonig, G. 1961. A modified procedure for lead staining of thin sections. *J. Biophys. Biochem. Cytol.* 11:736-739.
- Murthy, M. R. N., T. J. Reid III, A. Sicignano, N. Tanaka, and M. G. Rossmann. 1981. Structure of beef liver catalase. *J. Mol. Biol.* 152:465-499.
- Neupert, W., F. U. Hartl, E. A. Craig, and N. Pfanner. 1990. How do polypeptides cross the mitochondrial membranes? *Cell.* 63:447-450.
- Orr, E. C., G. C. Bewley, and W. C. Orr. 1990. cDNA and deduced amino acid sequence of *Drosophila* catalase. *Nucleic Acids Res.* 18:3663.
- Redinbaugh, M. G., G. J. Wadsworth, and J. G. Scandalios. 1988. Characterization of catalase transcripts and their differential expression in maize. *Biochim. Biophys. Acta.* 951:104-116.
- Robinson, P. A., B. H. Anderton, and T. L. F. Loviny. 1988. Nitrocellulose-bound antigen repeatedly used for the affinity purification of specific polyclonal antibodies for screening DNA expression libraries. *J. Immunol. Methods.* 108:115-122.
- Rosenkrantz, M., T. Alam, K. S. Kim, B. J. Clark, P. A. Srere, and L. P. Guarente. 1986. Mitochondrial and nonmitochondrial citrate synthases in *Saccharomyces cerevisiae* are encoded by distinct homologous genes. *Mol. Cell. Biol.* 6:4509-4515.
- Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1047-1081.
- Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC.)* 239:487-491.
- Schroeder, W. A., J. R. Shelton, J. B. Shelton, B. Robberson, G. Apell, R. S. Fang, and J. Bonaventura. 1982. The complete amino acid sequence of bovine liver catalase and the partial sequence of bovine erythrocyte catalase. *Arch. Biochem. Biophys.* 214:397-421.
- Silver, P. A. 1991. How proteins enter the nucleus. *Cell.* 64:489-497.
- Simon, M., G. Adam, W. Rapatz, W. Spevak, and H. Ruis. 1991. The *Saccharomyces cerevisiae* ADR1 gene is a positive regulator of transcription of genes encoding peroxisomal proteins. *Mol. Cell. Biol.* 11:699-704.
- Stone, D., and A. W. Phillips. 1977. The amino acid sequence of dihydrofolate reductase from L1210 cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 74:85-87.
- Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramani. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3255-3262.
- Vainshtein, B. K., W. R. Melik-Adamyani, V. V. Barynin, A. A. Vagin, A. I. Grebenko, V. V. Borisov, K. S. Bartels, I. Fita, and M. G. Rossmann. 1986. Three-dimensional structure of catalase from *Penicillium vitale* at 2.0 Å resolution. *J. Mol. Biol.* 188:49-61.
- Van Tuinen, E., and H. Riezman. 1987. Immunolocalization of glyceraldehyde-3-phosphate dehydrogenase, hexokinase, and carboxypeptidase Y in yeast cells at the ultrastructural level. *J. Histochem. Cytochem.* 35:327-333.
- Verner, K., and G. Schatz. 1988. Protein translocation across membranes. *Science (Wash. DC.)* 241:1307-1313.