Localization and targeting of isocitrate lyases in *Saccharomyces cerevisiae*

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Native isocitrate lyase from castor bean and a C-terminally truncated variant were expressed in *Saccharomyces cerevisiae* under the control of a galactose-inducible promoter. Both forms of isocitrate lyase were targeted to the yeast peroxisomes. They co-fractionated with catalase on sucrose-density-gradient centrifugation of a post-nuclear supernatant prepared from cells grown on oleic acid plus galactose, but were found in the cytosolic fractions when the cells were grown under conditions that repress peroxisome formation. The endogenous *S. cerevisiae* isocitrate lyase was found solely in the cytoplasmic fractions, even under growth conditions that induce peroxisome proliferation. This result shows that the presence of isocitrate lyase in peroxisomes is not essential for a functional glyoxylate cycle. Although the heterologous enzyme was transported to peroxisomes it was not enzymically active. Immunocytochemical studies provide independent evidence that the plant enzyme is imported into the matrix of yeast peroxisomes.

INTRODUCTION

Isocitrate lyase (ICL; *threo*-Ds-isocitrate glyoxylate-lyase, EC 4.1.3.1) is one of the enzymes of the glyoxylate cycle that enables plants and micro-organisms to accomplish a net synthesis of carbohydrate from two-carbon compounds [1]. ICL is generally found, along with other enzymes of the glyoxylate cycle and enzymes of the β-oxidation pathway, in specialized peroxisomes called glyoxysomes [2]. In higher plants these pathways play a crucial role in early post-germinative development when stored reserves of lipid are converted into carbohydrate to supply the demands of the young seedling before it becomes photosynthetically competent. In micro-organisms the same pathways permit the utilization of fatty acids (via acetyl-CoA) and other two-carbon compounds such as acetate and ethanol as carbon sources for growth.

Because peroxisomes lack DNA, all their protein constituents are the products of nuclear genes that are translated in, and imported from, the cytosol. So far, two pathways for the import of peroxisomal proteins have been elucidated by a combination of biochemical and genetic means [3]. One type of targeting signal, peroxisome targeting signal 1 (PTS-1), consists of a conserved tripeptide at the extreme C-terminus of the protein. In animals the consensus of this sequence is quite tightly defined: a serine, alanine or cysteine residue in the third last position; an arginine, lysine or histidine residue in the penultimate position; and a leucine (and possibly a methionine) residue in the final position [4,5]. In yeasts, and particularly in *Saccharomyces cerevisiae*, this consensus is much more divergent and includes sequences such as SKF and AKI [6,7]. Peroxisome assembly mutants have been characterized in a number of different yeasts [8–11]. Some of these (*pas*10, *S. cerevisiae*; *pas*8, *Pichia pastoris*) are specifically defective in the import of PTS-1-containing proteins [12,13]. Some human peroxisome assembly disorders also seem to be caused by an inability to import proteins with the PTS-1 signal [14].

The second import pathway is specified by a targeting signal known as PTS-2. This is found towards the N-terminus of a number of proteins although it does not have to be right at the N-terminus, unlike PTS-1, which does not function at internal locations. The consensus sequence for PTS-2 is RL/IXXXXXHL, where X is any amino acid [15–17]. A mutant of *S. cerevisiae* (*pas*7) has been characterized that is defective in the import of PTS-2-containing proteins, but is competent to import PTS-1 proteins [18]. This simple picture is complicated by the observation that some proteins have both PTS-1 and PTS-2 [19], whereas yet other proteins have additional, less well characterized signals that do not obviously fall into either class [6,20,21].

We are interested in the import pathway of ICL. We have studied the import of castor bean ICL into glyoxysomes isolated from sunflower cotyledons and in transgenic tobacco [22,23]. Castor bean ICL ends with the tripeptide ARM-COOH, which seems to be a reasonable fit with the PTS-1 consensus (although it is not known what the consensus is for plant peroxisomes). However, we can find no requirement for this tripeptide for targeting to peroxisomes either *in vitro* or *in vivo* ([22]; X. Gao and A. Baker, unpublished work), whereas others have obtained contradictory results with ICL from oilseed rape, which has the C-terminal tripeptide SRM-COOH [24]. To gain further insight into the mechanism of import of castor bean ICL, and to begin to compare the specificities of peroxisomal protein import between species, we expressed this protein in *S. cerevisiae* and analysed its subcellular distribution.

EXPERIMENTAL

Yeast strains and plasmids

S. cerevisiae strain BSL1-11B (MATa lys 2, his 4-519, ura 3Δ, leu 2-3, leu 2-122) was used in this study. The full-length cDNA encoding castor bean ICL was excised from pAB102 [22] as a

Abbreviations used: ICL, isocitrate lyase; PTS, peroxisome targeting signal.

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*Sac*I–*Pst*I fragment and inserted into the polylinker of pEMBLyex4 [25] to create pEMBLyex4-ICL, which placed the open reading frame under the control of a galactose-inducible promoter. The ICLΔ19 construct was prepared by insertion of a *Bgl*II linker (5′-TAGATCTA-3′) into a unique *Stu*I site within the coding region of castor bean ICL. This generated an in-frame stop codon resulting in the deletion of the last 19 residues of ICL. Plasmids were introduced into BSL1-11B by the lithium acetate transformation procedure [26].

Media and cell culture

Media used were WOYD [0.67 % (w/v) yeast nitrogen base with amino acids, 0.1 % (w/v) yeast extract, 0.3 % (w/v) glucose], WOYGal [0.67 % (w/v) yeast nitrogen base with amino acids, 0.1 % (w/v) yeast extract, 2 % (w/v) galactose], WOYOGal [0.67 % (w/v) yeast nitrogen base with amino acids, 0.1 % (w/v) yeast extract, 0.1 % (v/v) oleic acid, 0.1 % (v/v) Tween-20, 0.05 % (w/v) galactose], WOYO (as WOYOGal but without galactose), YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] and YPGal [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) galactose].

Subcellular fractionation

Single colonies were picked from SDC-ura plates [1 % (w/v) yeast nitrogen base without amino acids, 2 % (w/v) glucose, 0.5 % (w/v) casamino acids, 2 % (w/v) agar], and grown for 24 h in WOYD medium. Preculture (20 ml) was added to 200 ml of either WOYGal or WOYOGal medium and cultured for a further 24 h (except in the experiment shown in Figure 7, where culture in WOYOGal/WOYGal medium was for 16 h). The cells (0.5–1.2 g fresh weight) were collected by centrifugation, washed in water and resuspended in 0.1 M Tris/sulphate, pH 9.4, 10 mM dithiothreitol and incubated for 15 min at 30 °C. The cells were collected by centrifugation, washed in sorbitol buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4), resuspended in the same buffer and treated with 5 mg of Zymolyase 20000 per g of cells. Spheroplasts were recovered by centrifugation, washed once in breaking buffer (25 mM Mes/KOH, pH 6.0, 0.6 M sorbitol, 1 mM PMSF), resuspended in 2 ml of breaking buffer and homogenized with a hand homogenizer made of glass. The homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C, and 1 ml of the resulting post-nuclear supernatant was layered on to a 14 ml continuous (0.7–2.0 M) sucrose gradient. In addition to sucrose, the gradients also contained 10 mM Mes/KOH, pH 6.0, 10 mM KCl and 1 mM Na_2EDTA. The gradients were centrifuged for 5 h at 4 °C at 280000 ***g*** in a Beckman SW40 rotor (225000 ***g*** for the experiment shown in Figure 7 as the rotor was derated during the course of this work). Gradients were fractionated into 1 ml fractions with a peristaltic pump.

Biochemical analyses

For the preparation of whole-cell extracts, cells were cultured as for subcellular fractionation, then collected by centrifugation, resuspended in 1 ml of 0.1 % (v/v) potassium cholate and broken by vigorous vortexing in the presence of 1.5 g of glass beads (0.45–0.5 mm diameter). Glass beads and cellular debris were removed by centrifugation in an Eppendorf centrifuge and the supernatant was used for enzyme assays, protein determination and immunoblotting.

SDS/PAGE gels [10 % (w/v) acrylamide] were prepared and run essentially as described in [27] except that 2 % (v/v) polyacrylamide was included in the gel mix to provide greater mechanical stability. Silver staining was performed as described in [28]. Electrophoretic transfer of proteins to nitrocellulose was performed with an LKB 2117 Multiphor II apparatus. Primary antibodies were anti-(castor bean ICL) [29] and anti-(yeast mitochondrial hsp60) (a gift from Professor G. Schatz, Biozentrum, University of Basel, Basel, Switzerland). Primary antisera were used at a dilution of 1:20000 (ICL) or 1:5000 (hsp60). Immunodetection was performed with an enhanced chemiluminescence immunodetection kit (Amersham International) in accordance with the manufacturer's instructions. Protein was measured with the bicinchoninic acid protein assay kit (Pierce Chemical Company) with BSA as the standard. Catalase was assayed as described in [30]. The succinate dehydrogenase and ICL enzymic assays were performed as described in [31] and [2] respectively.

Immuno-electron microscopic analysis

Yeast cells transformed with pEMBLyexICL or with the vector alone and cultured in WOYOGal medium were fixed in 1.5 % (v/v) glutaraldehyde/2 % (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h. They were dehydrated in an ethanol series and embedded in freshly prepared LR White resin (medium grade; London Resin Co.). Ultrathin sections were cut with a Reichert Jung microtome Ultracut E, mounted on Formvar-coated nickel grids, blocked in 0.1 % (w/v) BSA in TBS buffer (20 mM Tris/HCl, 225 mM sodium chloride, 20 mM sodium azide, 0.5 % (v/v) Tween-20, pH 8.2) and incubated with a 1:500 dilution of anti-ICL antibodies in the blocking solution for 4 h at 0 °C. The grids were washed with five changes of TBS and then incubated with a 1:50 dilution of gold-conjugated goat anti-rabbit antibodies (15 nm gold particles; BioCell) in TBS buffer for 1 h at 0 °C followed by three washes with TBS buffer. Immunolabelled sections were double-stained with uranyl acetate and lead citrate, viewed and photographed with a transmission electron microscope (Philips EM 300).

RESULTS

Castor bean ICL is expressed in *S. cerevisiae* but is not enzymically active

BSL1-11B cells transformed with plasmid pEMBLyex4-ICL or the vector alone (pEMBLyex4) were grown overnight on oleate (WOYO medium), oleate plus 0.05 % galactose (WOYOGal medium) or 2 % galactose (WOYGal medium) as carbon source. Total protein was extracted from these cultures, separated by SDS/PAGE, transferred to nitrocellulose and probed with anti-(castor bean) ICL antibodies (Figure 1). In the control cells an immunoreactive protein slightly less than 66 kDa in molecular mass was detected in cultures grown on oleate and oleate plus 0.05 % galactose, but was virtually absent from cells grown on 2 % galactose. The endogenous *S. cerevisiae* enzyme behaves in this manner, being repressed by 2 % galactose but derepressed by a variety of non-fermentable carbon sources including oleate [32]. Galactose at a concentration of 0.05 % in the presence of oleate has been shown not to repress β-oxidation enzymes or peroxisome proliferation [33], and maximum derepression of ICL was seen by a combination of oleate and a small amount of fermentable carbon source, in that case 0.2 % glucose [32]. In the cells transformed with the castor bean ICL construct, a similar amount of ICL was seen in cells grown on oleate alone as in the control cells. Under these conditions the galactose-inducible promoter should have very low activity, and we are presumably detecting mostly or exclusively the endogenous enzyme. When 0.05 % galactose is included in the oleate-containing medium, a

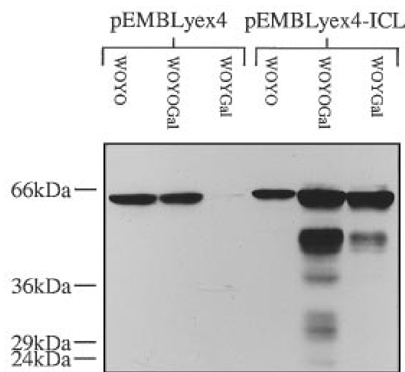

Figure 1 Expression of *S. cerevisiae* and castor bean ICL on different carbon sources

Cells were grown on the media indicated and crude protein extracts obtained as described in the Experimental section. Protein (50 μg) was loaded in each lane, separated by SDS/PAGE, transferred to nitrocellulose and detected with anti-(castor bean ICL) antibody.

Table 1 ICL specific activities from cells grown on different carbon sources

The samples from experiment 2 (marked with an asterisk) were used for the immunoblot shown in Figure 1.

Medium and experiment	pEMBLyex4-ICL activity (nmol/min per mg)	pEMBLyex4 activity (nmol/min per mg)
WOYO		
1	35.0	38.0
2	29.0*	34.0*
3	36.3	56.8
Mean ± S.D.	33.43 ± 3.89	42.93 ± 12.17
WOYOGal		
1	50.6	49.0
2	43.0*	16.2*
3	103.0	75.1
4	38.0	13.0
Mean ± S.D.	58.65 ± 30.02	38.33 ± 29.42
WOYGal		
1	15.8	16.4
2	6.0*	6.0*
3	33.0	19.06
Mean ± S.D.	18.27 ± 13.67	13.82 ± 6.90

large increase in the amount of immunoreactive material is seen, indicating the expression of the castor bean enzyme from the plasmid. In contrast with the control cells, a large amount of ICL antigen was seen in the culture grown on 2 % galactose, which represents the plasmid-encoded castor bean ICL. The lower-molecular-mass bands probably represent degradation products of ICL.

To determine whether the castor bean ICL was enzymically active, ICL-transformed and vector-transformed cells were grown on WOYGal, WOYO or WOYOGal medium as described above, crude protein extracts were obtained and ICL activity was measured. The results are given in Table 1. On WOYGal medium, where large amounts of the castor bean ICL protein were produced (Figure 1), the specific activities of ICL were very similar to those measured in the control samples. In the samples grown on WOYO medium, which results in derepression of the

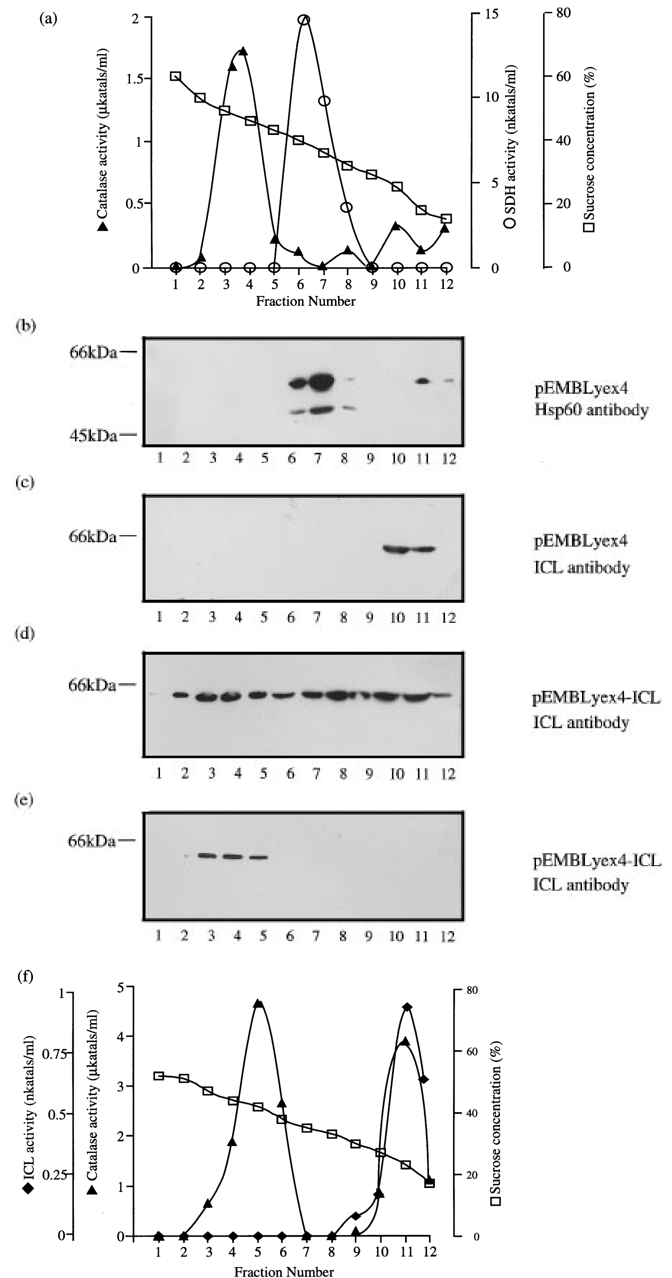

Figure 2 *S. cerevisiae* ICL is cytosolic whereas castor bean ICL is at least partly peroxisomal

Post-nuclear supernatants prepared from ICL-transformed or vector-transformed cells grown on 0.1 % oleate or 0.05 % galactose media were separated by centrifugation through continuous sucrose gradients: (**a**) the activities of catalase (recovery 86.5 %) and succinate dehydrogenase (recovery 92 %) in fractions from the control gradient; (**b**, **c**) immunoblots of the fractions from the control gradients probed with anti-yeast mitochondrial hsp60 (**b**) and anti-(castor bean ICL) (**c**); (**d**, **e**) fractions from the ICL transformed gradient probed with anti-castor bean ICL; (**f**) enzymic profiles of catalase and ICL in fractions from a different but otherwise identical gradient to that used for (**d**) and (**e**). Recoveries were 93.6 % for catalase and 126 % for ICL. In (**b**) to (**d**) equal volumes (200 μl) of the gradient fractions were loaded whereas in (**e**) 10 μg of protein was loaded in each lane.

endogenous *Saccharomyces* ICL (Figure 1), the specific activities were higher than in the samples grown on WOYGal medium but again there was little difference in activity between the cells expressing castor bean ICL and the controls (Table 1). The

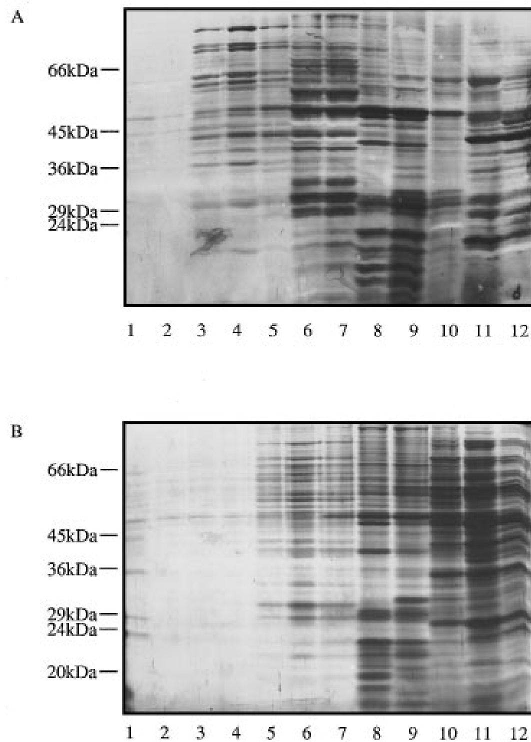

Figure 3 Polypeptide profile of gradient fractions from induced (A) and repressed (B) ICL transformants

Post-nuclear supernatants were prepared from cells grown on 0.1% oleic acid/0.05% galactose medium (induced) or on 2% galactose medium (repressed), and separated on sucrose gradients as before. Aliquots (200 μl) from each fraction were separated by SDS/PAGE and stained with silver. Fraction 1 is the bottom of the gradient and fraction 12 is the top.

results obtained for WOYOGal medium are less clear. Four separate experiments were conducted. In two cases there was little difference in specific activity (Table 1, experiments 1 and 3) and in two experiments the ICL transformants had 2.5–3-fold higher specific activity (Table 1, experiments 2 and 4). Means and standard deviations for all the results are shown in the table. The reason for the greater variablility between samples grown on WOYOGal medium is unknown, but the comparisons between the two strains grown on WOYO or WOYGal medium strongly suggest that the castor bean ICL, although expressed at a high level in the latter case, was not enzymically active. The results shown in Figure 2 also support this conclusion.

Castor bean ICL co-sediments with peroxisomes but the endogenous enzyme is cytosolic

To determine the subcellular localization of the castor bean ICL, post-nuclear supernatants from control and ICL-transformed cells that had been grown on oleic acid medium supplemented with 0.05% galactose (WOYOGal) were separated by centrifugation through continuous sucrose gradients. Fractions were taken and analysed by enzymic assay for the presence of catalase (a marker for peroxisomes) and succinate dehydrogenase (a marker for mitochondria). Additionally, fractions were blotted to nitrocellulose and probed with antibodies to yeast mitochondrial hsp60 and to castor bean ICL (Figure 2). The enzyme profiles in Figure 2(a) and immunoblots in Figures 2(b) and 2(c) are from vector-transformed control cells; the blots in Figures 2(d) and 2(e) and the enzyme data in Figure 2(f) are from ICL-transformed cells. The samples used in Figures 2(a), 2(b) and 2(c) were from the same gradient, as were the samples in Figures 2(d) and 2(e). The enzyme activities in Figure 2(f) were from a similar gradient to that shown in Figures 2(d) and 2(e).

Catalase and succinate dehydrogenase were clearly resolved from one another with peak activities in fractions 3 and 4, and 6 and 7 respectively (Figure 2a). hsp60 was detected in the same fractions as succinate dehydrogenase, as expected (Figure 2b). In the control cells the castor bean ICL antibody detected a protein of molecular mass slightly less than 66 kDa in the cytosolic fractions (Figure 2c). This is the endogenous *S. cerevisiae* ICL because it is seen in the vector-transformed cells and co-fractionates with ICL enzymic activity (Figure 2f). It is de-repressed by oleate but repressed by 2% galactose (Figure 1, and results not shown). In gradients prepared from the cells expressing castor bean ICL, immunoreactive material was found in all fractions of the gradient (Figure 2d). The material in the cytosolic fractions is probably a mixture of the *Saccharomyces* ICL and any castor bean ICL that had either failed to be imported or had leaked from damaged organelles. When the samples were loaded on an equal protein basis rather than by equal volumes, castor bean ICL was detected only in the gradient fractions that contained catalase, corresponding to the peroxisomal fractions (Figure 2e). Enzyme activity measurements on fractions from ICL-transformed cells did not detect any ICL activity in the peroxisomal fraction, only in the cytosolic fractions (Figure 2f). Fractions from vector-transformed gradients showed an identical profile of ICL activity (results not shown). Taken together, the results presented in Figures 1 and 2 and Table 1 lead us to conclude that castor bean ICL is specifically enriched in the peroxisomal fractions but is enzymically inactive, whereas the endogenous enzyme is cytosolic and active.

When peroxisome proliferation is repressed, castor bean ICL is found in the cytosolic fractions

Although some of the castor bean ICL co-sediments with the peroxisomal marker catalase, this might reflect the behaviour of heterogeneous aggregates of the enzyme rather than true import into yeast peroxisomes. If high levels of the castor bean ICL were expressed in the absence of peroxisome proliferation, aggregates would still form but the peroxisome peak would disappear. Thus under repressed conditions ICL should be found in the cytosolic fractions of the gradient if it is genuinely being imported, but the location should remain unchanged if the sedimentation behaviour is due to aggregation. Accordingly, ICL transformed cells were grown on WOYOGal medium (induced) or WOYGal medium (repressed) and fractionated on sucrose gradients as before. Aliquots of 200 μl from each 1 ml fraction was separated by SDS/PAGE and stained with silver (Figure 3). Fractions 3 and 4, which correspond to the peroxisomal peak in the induced sample, are almost devoid of protein in the repressed sample, demonstrating the shift of peroxisomal components to less dense fractions under these conditions.

Figure 4 shows the results of Western blots and enzymic assays performed on these gradient fractions. As before, catalase is found in the denser region of the gradient from the oleate-grown cells. Castor bean ICL again co-fractionated with catalase (Figure 4c) and away from the mitochondrial marker hsp60 (Figure 4b). In marked contrast, when catalase was measured in fractions from the gradient of the repressed cells, it was found in the cytosolic fractions (Figure 4d). Note also the much lower activity of catalase in the repressed cells. This might reflect the activity of

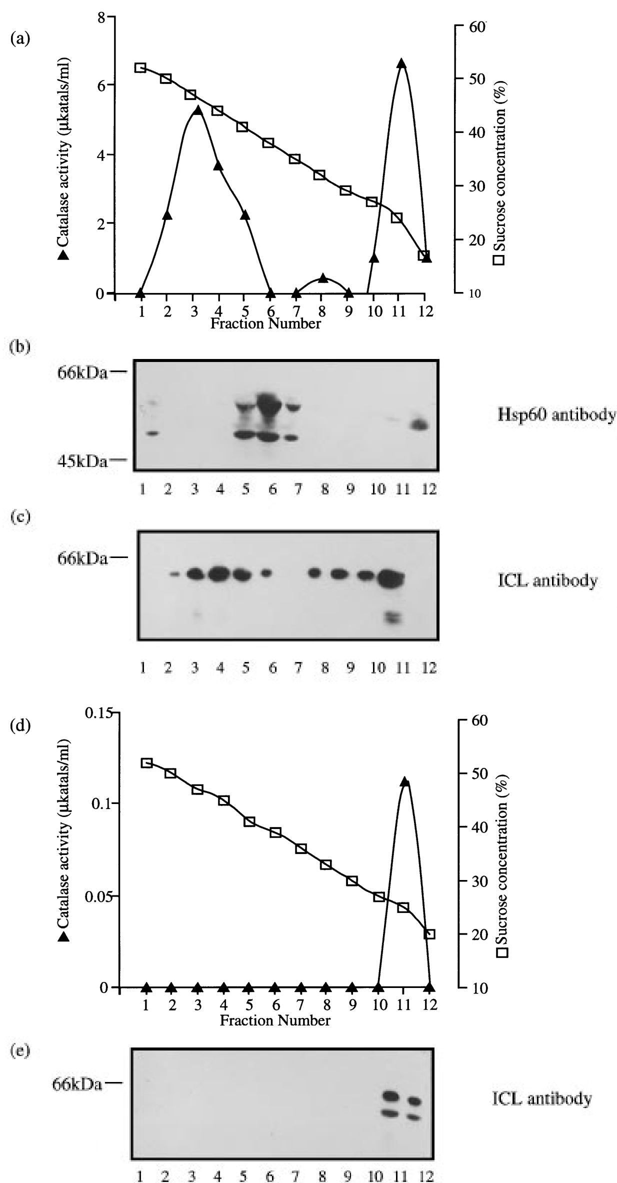

Figure 4 Castor bean ICL is found in the cytosol when cells are grown under peroxisome-repressing conditions

Samples from the induced (**a–c**) and repressed (**d**, **e**) gradient fractions shown in Figure 3 were subjected to enzymic assay and immunoblotting. (**a**, **d**) Catalase activity; (**b**) hsp60 antibody. Catalase recoveries: in (**a**), 90%; in (**d**), 17%. Note that the activity of catalase under repressed conditions is extremely small and is sometimes undetectable. (**c**, **e**) Castor bean ICL antibody. Equal volumes (100 μl) of each fraction were used for the immunoblots.

the cytosolic catalase isoenzyme, catalase T. When fractions from the repressed gradient were blotted for ICL, immunoreactive material was detected only in the cytosolic fractions (Figure 4e). There was much less immunoreactivity than in the blots from the induced cells, and some evidence of degradation in the form of lower-molecular-mass fragments, which were detected by the antibody. Thus ICL co-fractionates with the peroxisomal marker catalase when peroxisomes are induced, but is found in the cytosol and degraded under conditions where peroxisomes are repressed.

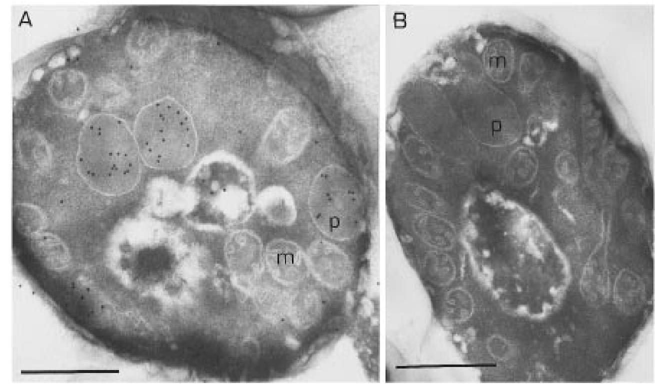

Figure 5 Immuno-electron micrographs of ICL localization

(**A**) pEMBLyexICL-transformed cells probed with anti-ICL antiserum; (**B**) pEMBLyexICL-transformed cells probed with preimmune serum. Scale bars, 0.5μm. Abbreviations: p, examples of peroxisomes; m, examples of mitochondria.

Castor bean ICL is found in the peroxisomal matrix

To determine whether castor bean ICL was completely imported into the matrix of *S. cerevisiae* peroxisomes and not just associated with the surface, an immuno-electron microscopic localization study was performed. Samples of cells expressing castor bean ICL (Figure 5), or cells expressing the endogenous ICL only (vector-transformed cells; results not shown) were incubated with anti-(castor bean ICL) antiserum or preimmune serum followed by gold-conjugated goat anti-rabbit IgG. Cells expressing castor bean ICL showed prominent labelling over peroxisomes with little labelling over other cellular structures (Figure 5A). In contrast, preimmune serum gave no labelling over peroxisomes or any other cellular structure (Figure 5B). A statistical analysis of the immunolabelling results is presented in Table 2. In cells expressing castor bean ICL incubated with antiserum, 41 out of 52 peroxisome profiles counted (79 %) had gold particles, with a mean of 3.9 gold particles per peroxisome; individual peroxisomes had up to 14 gold particles. In contrast, no labelled peroxisomes (out of 35) were seen in samples incubated with preimmune serum or in vector-transformed cells. Labelling of mitochondria was nearly two orders of magnitude lower and similar between control cells and those expressing castor bean ICL. These results demonstrate the specificity of the peroxisomal labelling. Cytosolic labelling was low and similar in both control cells and cells expressing castor bean ICL. In each case, less labelling was seen with preimmune serum, but the level of labelling was too low to draw definite conclusions about the location of *S. cerevisiae* ICL with this technique. However, the sucrose gradient results (Figures 2c and 2f) argue strongly that *S. cerevisiae* enzyme is cytosolic.

Targeting of ICL

Why should the same enzyme from different species have different subcellular localizations? If the sequences of *S. cerevisiae* ICL and castor bean ICL are compared, the most obvious difference is that the castor bean enzyme contains a C-terminal extension ending in the putative PTS-1 sequence ARM. In contrast, the *S. cerevisiae* enzyme ends with the tripeptide VKK, which is not known to be a functional PTS-1 in *S. cerevisiae* (Figure 6). To investigate whether this C-terminal extension is responsible for the difference in behaviour between the two ICL species, a mutant version of the castor bean ICL lacking the last 19

Table 2 Statistical analysis of ICL immunolabelling

The numbers of gold particles associated with mitochondria, peroxisomes and cytosol were determined from photomicrographs of 28 whole cells of the ICL transformant and 14 whole cells of the vector-transformed control, both labelled with anti-ICL antiserum. Results from three independent identical grids were pooled in each case. In a similar manner gold particles were scored for sections cut from the same blocks and incubated with preimmune serum under the same conditions. These results are from a single grid in each case. Results are means ± S.D.; numbers in parentheses are the total numbers of organelles scored for that data set.

	pEMBLyex4-ICL		pEMBLyex4	
Cellular structure	Anti-ICL	Preimmune	Anti-ICL	Preimmune
Peroxisomes	3.90 ± 4.00 (52)	0.00 (12)	0.00 (18)	0.00 (5)
Mitochondria	0.05 ± 0.29 (285)	0.00 (82)	0.07 ± 0.26 (98)	0.00 (24)
Cytosol	2.04 ± 2.12 (28)	0.43 ± 0.79 (7)	2.21 ± 1.53 (14)	0.33 ± 0.47 (3)

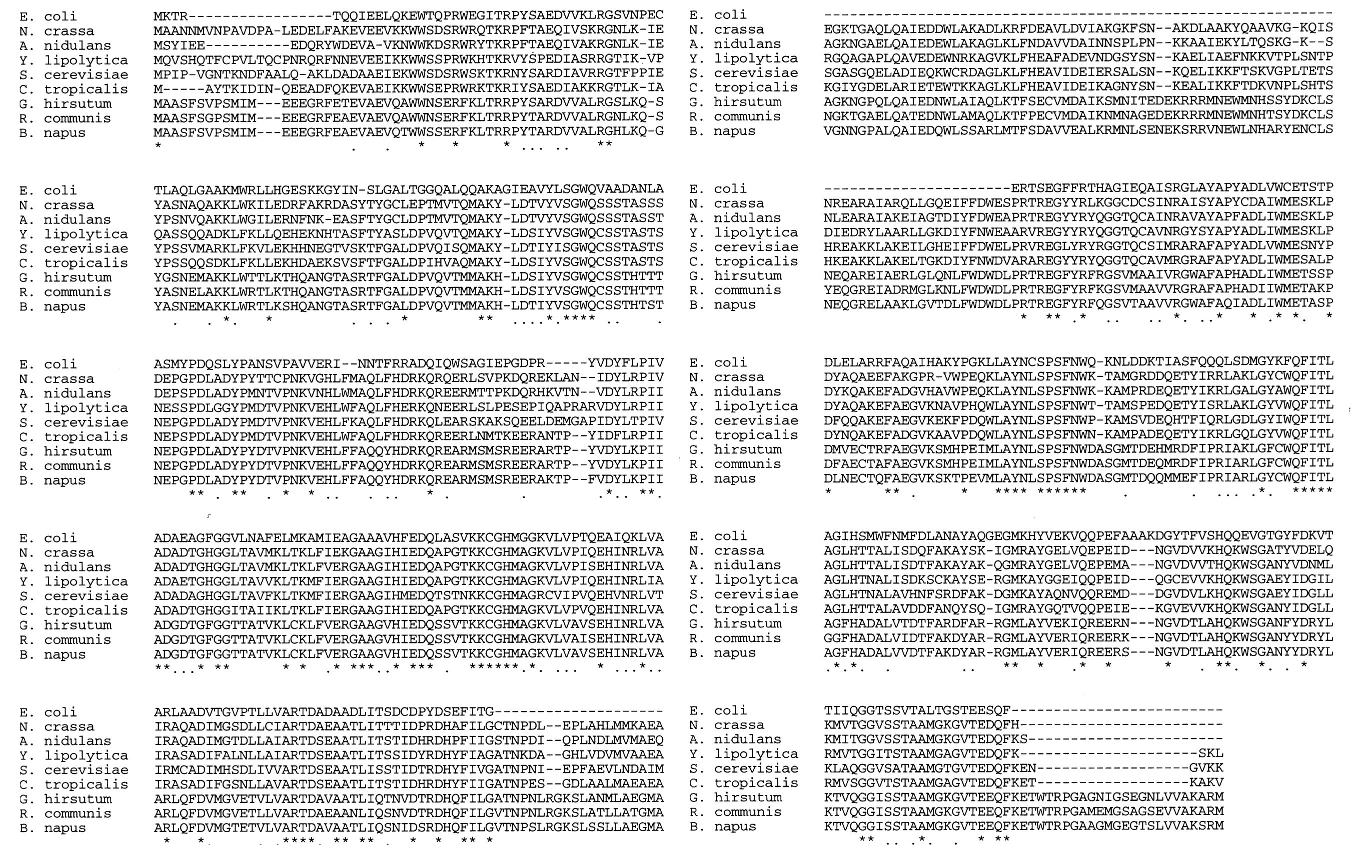

Figure 6 Sequence alignments of ICL from various organisms

ICL sequences from *E. coli* (ACEA-ECOLI), *N. crassa* (ACEA-NEUCR), *Aspergillus nidulans* (ACA-EMENI), *Y. lipolytica* (YLICL1A), *S. cerevisiae* (ACEA-YEAST), *C. tropicalis* (ACEA-CANTR), *Gossypium hirustum* (ACEA-GOSHI), *Ricinus communis* (ACEA-RICCO) and *Brassica napus* (ACEA-BRANA) were taken from the SWISS-PROT database and aligned by using the program ClustalV. Conserved residues are indicated with an asterisk; conserved substitutions with a dot.

residues was expressed in *S. cerevisiae* and cell fractionation studies were performed as before. The distribution of ICLΔ19 in a sucrose gradient (Figure 7b) is virtually identical with that seen with the full-length castor bean ICL (Figure 2d) and the lower peak coincides with the location of catalase in the gradient (Figure 7a). In this experiment the resolution between the peroxisomal and mitochondrial peaks was not as good as in the previous experiments and there was overlapping of the markers. This is probably due to the shorter peroxisome induction period and the lower centrifugation speed used in this experiment (see the Experimental section). However, when the cells expressing ICLΔ19 were cultured on 2% galactose, ICLΔ19, like full-length ICL, was found in the cytosolic fractions and was partly degraded (compare Figures 7d and 4e), whereas the position of the mitochondrial marker hsp60 was unaltered by growth on galactose (compare Figures 7c and 7e). This provides strong evidence that ICLΔ19 is also capable of being imported into peroxisomes of *S. cerevisiae*.

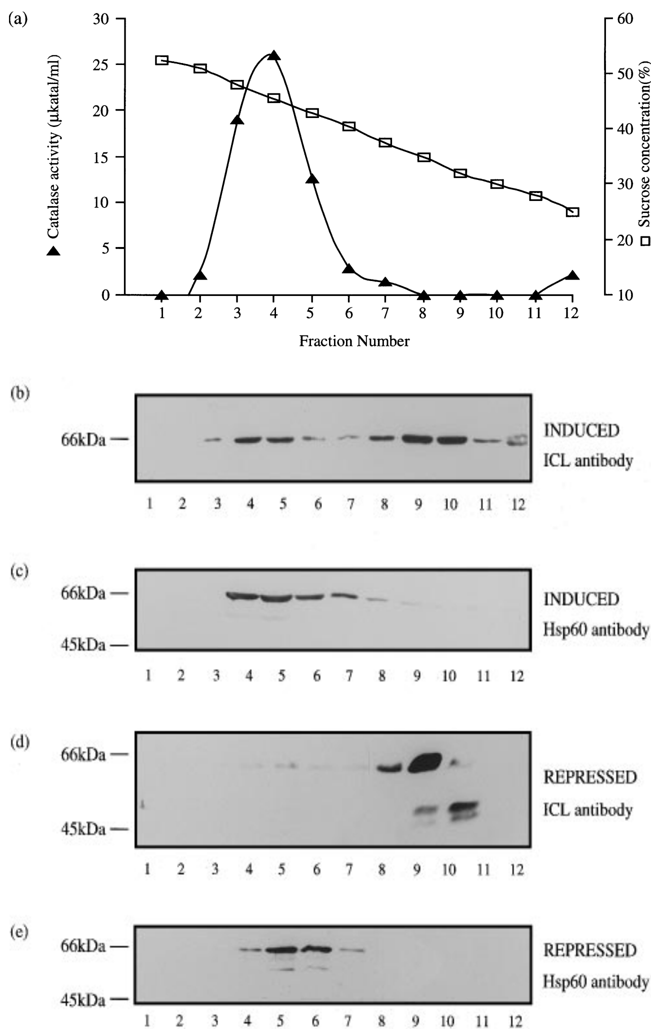

Figure 7 The ICLΔ19 mutant is still targeted to peroxisomes

Post-nuclear supernatants from cells transformed with ICLΔ19 in pEMBLyex4 that had been grown on 0.1% oleate/0.05% galactose (**a**, **b**, **c**) or 2% galactose (**d**, **e**) were separated on sucrose gradients: (**a**) catalase activity (recovery 88%); (**b**, **c**) immunoblots of fractions from the same gradient probed with anti-ICL (**b**) and anti-hsp60 (**c**); (**d**, **e**) immunoblots of fractions from the same gradient of galactose-grown cells probed with anti-ICL (**d**) and anti-hsp60 (**e**).

DISCUSSION

The results presented in this paper provide clear evidence for the targeting of castor bean ICL to yeast peroxisomes, although it is not enzymically active. The lack of enzymic activity could be due to differences in post-translational regulatory mechanisms. The activity of ICL in both *E. coli* and *S. cerevisiae* is subject to regulation by phosphorylation, but in different ways. In *E. coli* the enzyme is activated by phosphorylation on a histidine residue or residues [34], whereas in *S. cerevisiae* the addition of glucose to ethanol-grown cultures leads to phosphorylation of the enzyme and loss of activity [35]. The plant enzymes lack the putative phosphorylation sites found in the *Saccharomyces* [36] and *E. coli* [37] enzymes. Indeed, in the latter study no evidence could be found for phosphorylation of watermelon ICL during germination. Thus if the castor bean ICL is subject to post-translational regulatory mechanisms, these might not be operating in the heterologous yeast host. In contrast, *Candida tropicalis* ICL was active when expressed in *S. cerevisiae* [38]. Alcohol oxidase of *Hansenula polymorpha*, when expressed in *S. cerevisiae*, was also enzymically inactive; in that case the enzyme failed to oligomerize [39]. Like the *Saccharomyces* enzyme castor bean ICL is a tetramer, but we have not investigated its assembly in yeast.

In our studies we could find no evidence for the association of the endogenous *Saccharomyces* enzyme with peroxisomes. In this respect our observations are in complete agreement with those of McCammon et al. [40], who recovered only 0.2% of total ICL activity with the peroxisomal fraction despite obtaining 65–96% of β-oxidation enzymes in these fractions. They also obtained low recovery of another glyoxylate cycle enzyme, malate synthase, finding 10–15% of activity in peroxisomes, and speculated that certain enzymes could be selectively lost from damaged organelles [40]. Peroxisomes from many sources contain a highly structured matrix core. In spinach leaf peroxisomes this is sufficiently stable for many of the enzymes within it to exhibit structure-linked latency even in the absence of a boundary membrane [41]. One might imagine that enzymes firmly bound to such a core might be less prone to leakage from damaged organelles than those with a looser association; however, it seems unlikely that a heterologous protein, the castor bean ICL, would be more tightly associated with any such matrix core than with the endogenous protein. Neither ICL antigen nor ICL activity was detected in the peroxisomes by the very sensitive ECL detection method, by enzymic assay or by electron microscopy. We therefore conclude that *S. cerevisiae* ICL is a cytosolic enzyme. In higher plants another enzyme of the glyoxylate cycle, aconitase, is cytosolic [42].

The *S. cerevisiae pas* mutants originally isolated were competent to grow on acetate (which presumably requires ICL activity) even though many peroxisomal proteins are relocated to the cytosol [8]; likewise *PER* mutants of *H. polymorpha*, which are devoid of peroxisomes, are still able to utilize ethanol [9]. Thus there seems to be no strong argument against ICL's being functional and able to support glyoxylate cycle activity in the cytosol. Indeed if ICL were originally peroxisomal in some 'wild-type' ancestor of laboratory yeasts there might not have been any strong selective pressure to retain a peroxisomal location under laboratory conditions. By analogy, on screening for *pas* mutants Tabak and co-workers found that one of their 'wild-type' yeast strains was in fact a *pas* mutant [12]. To exclude the possibility that we were working with a strain with a mutant ICL that is no longer capable of import, we examined the location of ICL in a different strain (UTL7A) by sucrose gradient analysis and found it to be cytosolic as well (results not shown). Together with the results of McCammon et al. [40], our results provide compelling evidence for a cytosolic location for *S. cerevisiae* ICL. The evidence for ICL's being glyoxysomal in other fungi such as *Neurospora crassa* and *C. tropicalis* is much stronger [43,44]. Indeed the *Candida* enzyme can be imported into peroxisomes when expressed in *Saccharomyces* [38].

Comparisons of sequence alignments of the different ICL proteins (Figure 6) do not provide any easy clues to this puzzle. The plant enzymes all have a C-terminal extension of 18–24 amino acids compared with the C-termini of the fungal proteins, and end with a putative PTS-1. However, castor bean ICL does not need this sequence for import into plant peroxisomes, either *in vitro* [22] or in transgenic plants (X. Gao and A. Baker, unpublished work) or, as reported here, in yeast. The *Candida* enzyme ends with the tripeptide AKV-CO_2H, which is rather similar to AKI-CO_2H, the PTS for the *C. tropicalis* trifunctional enzyme and which also works in *S. cerevisiae* [7], and the *Yarrowia lipolytica* ICL ends with the well characterized PTS sequence SKL-CO_2H [45] but the *Saccharomyces* protein ends

with VKK-CO_2H, which has not been shown to be a PTS. The *Neurospora* and *Aspergillus* proteins lack even this and are only one or two amino acids longer than the *E. coli* ICL. This implies that *Neurospora* and *Aspergillus* ICL species do not use a PTS-1 mechanism for import and that the castor bean enzyme is not dependent on a PTS-1 for import. Whether *Neurospora*, *Aspergillus* and castor bean ICL share an alternative targeting signal remains unknown.

The finding that the castor bean ICL targets to peroxisomes, whereas the endogenous enzyme apparently does not, opens up the possibility of using *pas* mutants of *S. cerevisiae* to investigate further the targeting signal used to direct the protein to peroxisomes in yeast. *pas*7 displays a selective import defect for proteins with PTS-2, whereas *pas*10 fails to import proteins with PTS-1. Expression of the castor bean enzyme in these strains might provide insight as to whether the protein is being imported by either of these two pathways or by a third, as yet uncharacterized, pathway. Sequence swap experiments between the castor bean and *Saccharomyces* enzymes might also help to pinpoint the targeting sequences.

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