Mutagenesis of the Amino Targeting Signal of *Saccharomyces cerevisiae* 3-Ketoacyl-CoA Thiolase Reveals Conserved Amino Acids Required for Import into Peroxisomes in Vivo*

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*Saccharomyces cerevisiae* peroxisomal 3-ketoacyl-CoA thiolase is a soluble matrix protein that does not end in a consensus peroxisomal targeting signal-1. The amino terminus of *S. cerevisiae* peroxisomal thiolase is conserved in 6 of 11 residues with the amino terminus of rat thiolase B, shown to act as a peroxisomal targeting signal-2 (Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. (1991) *EMBO J.* 10, 3255-3262). Unlike mammalian peroxisomal thiolases, there is no extensive cleavage of *S. cerevisiae* thiolase upon import into peroxisomes. We demonstrate by *in vivo* expression that the amino-terminal 16 amino acids of *S. cerevisiae* thiolase are necessary and sufficient for targeting to peroxisomes. This result implies that yeast, like mammalian cells, can target proteins to the peroxisomal matrix by at least two different routes. We also demonstrate by targeted mutagenesis and *in vivo* expression of mutated thiolase genes that three amino acids conserved in the amino termini of all known thiolases are critical for efficient targeting of *S. cerevisiae* thiolase to peroxisomes.

The correct targeting of proteins to the peroxisome is essential for its biogenesis. The features of protein targeting to peroxisomes are essentially similar to those involved in the assembly of other organelles (for a review, see Aitchison et al. (1992)). As in proteins targeted to the mitochondrion and the chloroplast, peroxisomal proteins contain signals that are required for targeting to the peroxisome and for initiating an interaction with a putative proteinaceous import apparatus (McCollum et al., 1993).

A subset of soluble matrix peroxisomal proteins is targeted by a carboxy-terminal tripeptide motif designated peroxisomal targeting signal-1 (PTS-1)\(^1\) typified by the Ser-Lys-Leu of firefly luciferase (Gould et al., 1987). In contrast, the PTS of the rat peroxisomal matrix enzyme 3-ketoacyl-CoA thiolase is contained within the first 11 amino acids of a cleavable sequence located at its amino terminus (Swinkels et al., 1991; Osumi et al., 1991).

\(^1\) The abbreviation used is: PTS, peroxisomal targeting signal.

The amino-terminal 11 amino acids of *Saccharomyces cerevisiae* thiolase are identical in 6 of 11 residues with the PTS-2 of rat peroxisomal thiolase. A comparison of the amino-terminal regions of mammalian and yeast peroxisomal thiolases reveals three amino acids identical in all thiolases (Fig. 1). Because of these similarities, we wished to determine whether the amino terminus of *S. cerevisiae* thiolase functions as a PTS-2. We report that the amino-terminal 16 amino acids of *S. cerevisiae* thiolase act as a PTS-2 and that the arginine at position 4 and the leucines at positions 5 and 12 are critical for efficient targeting of thiolase to peroxisomes.

**MATERIALS AND METHODS**

*Culture Media*—Yeast strains carrying plasmids were cultured and maintained in YNBD (0.67% yeast nitrogen base without amino acids (YNB), 2% glucose). To induce the expression of genes encoding peroxisomal proteins and to proliferate peroxisomes, cells grown in YNB were pelleted, washed in sterile water, and transferred to SCIM (0.67% YNB, 0.05% yeast extract, 0.5% peptone, 0.1% (w/v) Tween 40, 0.1% glucose, 0.1% (w/v) oleic acid). To test for growth on oleic acid-agar, precultures grown overnight in YNBD were diluted 1:10 with sterile water, and 2 μl was applied as a droplet to the surface of YNO plates (0.67% YNB, 0.05% yeast extract, 0.5% (w/v) Tween 40, 0.1% (w/v) oleic acid; Erdmann et al. (1989)). For transformation, yeast strains were cultured in YEPD (1% yeast extract, 2% peptone, 2% glucose). Media were supplemented with histidine and uracil each at 20 μg/ml, as required.

*Transformation of Yeast*—Plasmid DNA was introduced into 20-μl aliquots of cells by electroporation with a BRL Cell-Portator equipped with a voltage booster set to 4-ka resistance and delivering a pulse with a field strength of approximately 7.5 kV/cm (Nutney et al., 1993). Cells were diluted into 100 μl of 1 M sorbitol and spread onto selective YNBD agar plates.

*Yeast Strains*—The DL-1 strain of *S. cerevisiae* (MATa, leu2, ural3, his3, URA3) carrying its nuclear thiolase gene (THI) disrupted by homologous recombination with the URA3 gene. A HindIII fragment containing the URA3 gene from the plasmid YCp50 was made blunt with the Klenow fragment of DNA polymerase and inserted by ligation into pGEM7Zf(+) cut with SmaI to yield pGEMURA3. The THI gene was excised from pSG524 as a BamHI-XbaI fragment and subcloned between the BamHI-XbaI sites of a derivative pGEM7Zf(+) with its SpeI site destroyed. The THI coding sequence between codons Cys-107 and Leu-359 was removed by digestion with SpeI and ClaI. This portion was replaced with an SpeI-ClaI fragment from pGEMURA3 containing the URA3 gene to yield pSTUD3. pSTUD3 was digested with BamHI and SalI, and the resulting fragment (Fig. 2, STUD) was introduced into DL-1 cells by electroporation. Colonies capable of growth on glucose in the absence of uracil but incapable of growth on oleic acid when replicated onto YNO were subjected to further testing by Southern and Western blot analysis to confirm the success of the disruption of the THI gene.

*Thiolase Expression Constructs*—The *S. cerevisiae* THI gene was expressed from plasmids pSG524 and pSG522 (Fig. 2) under the control of the oleic acid-responsive *S. cerevisiae* fatty acyl-CoA oxidase promoter (Wang et al., 1990) in the plasmid pBS156 (Sikorski and Hieter, 1989). To synthesize thiolase initiating at the AUG encoding Met-17, pSG522
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**RESULTS**

**Thiolase of *S. cerevisiae* Is Not Cleaved—**Forms A and B of rat peroxisomal thiolase and human peroxisomal thiolase are cleaved upon import into peroxisomes (Hijikata et al., 1987; Tager et al., 1985). Cleavage results in mature forms of thiolase that are substantially shorter than their newly synthesized counterparts. *S. cerevisiae* peroxisomal thiolase synthesized *in vitro* had the same electrophoretic mobility in SDS-polyacrylamide gel electrophoresis as thiolase from purified peroxisomes (Fig. 3). This result suggests that thiolase is not cleaved upon import into peroxisomes of *S. cerevisiae*. However, because of the limited resolution of this approach, removal of a few amino acids from the newly synthesized thiolase upon import into peroxisomes cannot be ruled out. Nevertheless, *S. cerevisiae* thiolase is imported into peroxisomes without the extensive cleavage observed for mammalian peroxisomal thiolases.

**The Targeting Signal of *S. cerevisiae* Peroxosomal Thiolase Resides at Its Amino Terminus—**A mutated gene (Fig. 2, pSG522ΔNcoI) coding for a truncated form of *S. cerevisiae* peroxisomal thiolase lacking the first 16 amino acids at its amino terminus was expressed in the THI gene disruption strain, STUD. This truncated thiolase failed to support growth on oleic acid-agar (Fig. 4, A1–16) and was localized to the cytosol (Fig. 5, A1–16). Thiolase A1–16 retained enzymatic activity (Table I), indicating that the inability to grow on oleic acid-agar was due not to a loss of enzymatic activity by the truncated thiolase but to its lack of import into peroxisomes. Moreover, since the truncated thiolase retained normal levels of enzymatic activity, its

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**Subcellular Fractionation—**Yeast cells grown in SCIM were harvested and converted to spheroplasts by digestion with Zymolyase. Transformants were checked for growth on oleic acid-agar, and the transformant was prepared and recentrifuged at 20,000 g for 20 min to obtain a pellet (20 kgP) enriched for peroxisomes and mitochondria and a supernatant (20 kgS) enriched for cytosol (Atchison et al., 1992). Cytosolic contamination of the 20 kgP was less than 10%, as measured by the cytosolic marker enzyme inorganic pyrophosphatase. A portion of the 20 kgP was further fractionated to yield a purified peroxisomal fraction on a gradient (1.3 ml of 1%, 2.5 ml of 28%, 0.7 ml of 35%, and 0.5 ml of 50% (w/v) Nycodenz dissolved in disruption buffer) centrifuged in a Beckman VT65 rotor at 36,000 rpm for 70 min at 4 °C (Lewin et al., 1990). In subcellular fractions of yeasts expressing gGPrA and the thiolase-gGPrA fusion, protease inhibitors including leupeptin, chymostatin, pepstatin, and antipain each at 2.5 μg ml⁻¹, N-α-tosyl-l-lysine chloromethyl ketone at 4 μg ml⁻¹, aprotonin at 50 μg ml⁻¹, 1 mm phenylmethylsulfonyl fluoride, and 2.5 μg NaF were included in the Zymolyase and homogenization buffers.

**Thiolase Assay—**Yeast cells grown in SCIM were pelleted and disrupted with glass beads in buffer containing 50 μM Tris-HCl (pH 7.5), 50 μM NaCl, 0.1 μM EDTA, 0.1 mM ZnCl₂, and 1 mM phenylmethylsulfonyl fluoride (Needleman and Tzagoloff, 1975). Thiolase activity was assayed as described by Suebert et al. (1968) except that enoyl-CoA hydratase was not required for the enzymatic formation of the substrate. 3-Hydroxydecanoyl-CoA was added at a final concentration of 50 μM to a solution of 50 μM Tris-HCl (pH 9.0), 50 mM KCl, 25 mM MgCl₂, 50 μg ml⁻¹ bovine serum albumin, 1 mM NAD, 1 mM sodium pyruvate, 25 micromol of pig heart 3-hydroxyacyl-CoA dehydrogenase, 1.8 units of rabbit muscle lactate dehydrogenase. The formation of 3-ketodecanoyl-CoA was monitored as an increase in absorbance at 303 nm. Measurement of thiolase activity was initiated by the addition of cell lysate containing 1% (w/v) Triton X-100 and of CoASH to a final concentration of 150 μM. Activity was calculated using the extinction coefficient ε = 13.9 cm² μmol⁻¹.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—**SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gels (Laemmli, 1970). Western blotting was performed essentially as described (Burnette, 1981). Nitrocellulose was blocked with 8% skim milk in 0.05 M Tris-HCl (pH 7.4) and 0.5% Tween 20. Thiolase was detected with rabbit anti-thiolase serum, followed by 125I-Protein A (Amersham). The gGPrA-based passenger protein was detected with 125I-labeled rabbit IgG. Blots were exposed to X-Omat AR film or to a storage phosphor screen, which was scanned on a PhosphorImager and quantified using software provided by the manufacturer (Molecular Dynamics). Nytran membrane (Schleicher and Schuell) was used to compare the electrophoretic mobilities of *in vitro*-translated thiolase and thiolase isolated from peroxisomes. The membrane was first probed with anti-thiolase serum followed by 125I-Protein A. After washing, the blot was dried, soaked in 10% 2,5-diphenyloxazole in toluene, and exposed to X-Omat AR film at -70 °C.

**In Vivo Transcription and Translation—**pGEM7Zf(+) containing a BamHI-XbaI insert encoding thiolase was linearized with XbaI and transcribed using SP6 RNA polymerase. The RNA was translated in rabbit reticulocyte lysate in the presence of [35S]methionine.
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Fig. 2. Schematic illustration of constructs. The filled segment represents that part of the S. cerevisiae gene encoding the amino-terminal 16 amino acids of peroxisomal thiolase. The large open segment represents the rest of the THI gene coding region. The small open segments represent restriction endonuclease fragments contained within the large open segment. The gray segment represents the region coding for the IgG-binding domains of protein A. The finely hatched segment encodes the amino-terminal 27 amino acids of chimpanzee globin modified to contain a glycosylation site. The boldly hatched segment indicates the HindIII fragment of S. cerevisiae URA3. The arrow indicates the direction of transcription of the URA3 gene. ATG, initiation codon of wild-type thiolase; B, BamHI; N, NcoI; X, XhoI; E/X, EcoRV fused to an XbaI site made blunt with the Klenow fragment of DNA polymerase I. Illustrations are not to scale.

Fig. 3. S. cerevisiae thiolase is not cleaved upon import into peroxisomes. S. cerevisiae peroxisomes and in vitro translated S. cerevisiae peroxisomal thiolase were separated by SDS-polyacrylamide gel electrophoresis, transferred to Nytran membrane, and detected as described under "Materials and Methods." Lane PXM, 5 μg of S. cerevisiae peroxisomes. Lane IVT, in vitro-translated S. cerevisiae peroxisomal thiolase. Arrows indicate migrations of molecular size markers (in kDa).

inability to be imported into peroxisomes was probably not the result of a gross alteration of structure but rather the result of the removal of the peroxisomal targeting signal of thiolase.

The amino-terminal 16 amino acids of thiolase were fused to the gGPrA passenger protein to show their sufficiency for import into peroxisomes. This passenger has been shown to be cytosolic in the absence of added targeting information (Janiak et al., 1994). However, it is passive to translocation and is correctly targeted by both carboxyl-terminal and amino-terminal signals to a variety of cellular compartments, including the yeast peroxisome (Janiak et al., 1994). The gGPrA passenger encoded by pgGPrASTOP (Fig. 2) was exclusively localized to the cytosol (Fig. 6, panel A, upward pointing arrowheads). The fusion protein (Fig. 2, gGPrASCTN) composed of the amino-terminal 16 amino acids of thiolase attached to the amino terminus of gGPrA (Fig. 6, panels B and C, upward pointing arrowheads) co-fractionated with endogenous thiolase (Fig. 6, downward pointing arrowheads). The thiolase-gGPrA fusion construct was released from the organelle pellet by treatment with the detergent Triton X-100, indicating that the thiolase-gGPrA fusion protein is targeted and imported into peroxisomes and does not form an insoluble inclusion body (data not shown; McCollum et al. (1993)).

Mutations in Several Conserved Amino Acids within the Amino-terminal PTS of S. cerevisiae Thiolase Impede or Abolish Import into Peroxisomes—A comparison of peroxisomal thiolas reveals a conservation among all thiolas of certain amino acids found within the 11-amino acid PTS of rat thiolase (Swinkels et al., 1991; Osumi et al., 1991). In S. cerevisiae peroxisomal thiolase, these conserved residues are Arg-4, Leu-5, and Leu-12 (Fig. 1). Gln-6 and His-11 are conserved among S. cerevisiae thiolase and the mammalian thiolas, but they are not conserved in the thiolas from other organisms. The importance of these amino acids in targeting S. cerevisiae thiolase to peroxisomes was determined by random mutagenesis of the corresponding codons followed by expression of the mutant thiolase genes in vivo. Subcellular localization of mu-
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The percent distribution of thiolase in the 20 kgP and 20 kgS was determined by Western blot analysis with 125I-protein A, followed by quantitation on a phosphorimager. Growth on YNO agar was assessed after incubation at the indicated temperature for 10 days. Enzyme activity was determined as described under “Materials and Methods” (cultured at 30 °C).

| Strain | 20 kgS | 20 kgP | Growth at 223 °C | Growth at 30 °C | Activity nmol min⁻¹ mg protein⁻¹ |
|--------|--------|--------|------------------|-----------------|--------------------------------|
| null   | ND*    | ND     | -                | -               | ND                             |
| WT     | 15     | 85     | +                | +               | 6.6                            |
| Δ1–16  | 97     | 5      | +                | +               | 4.7                            |
| S2A    | 14     | 86     | +                | +               | 4.9                            |
| R4S    | 90     | 10     | +                | +               | 6.5                            |
| R4K    | 65     | 35     | +                | +               | 10.3                           |
| R4G    | 95     | 5      | +                | -               | 8.0                            |
| L4/L12M| 14     | 86     | +                | +               | 7.7                            |
| L5P    | 90     | 10     | +                | +               | 10.9                           |
| L5R    | 88     | 12     | +                | -               | 0.14                           |
| L5V/Q6P| 92     | 8      | +                | -               | 10.7                           |
| Q6P    | 70     | 20     | +                | +               | 8.2                            |
| Q6H    | 24     | 76     | -                | +               | 11.0                           |
| H11L   | 27     | 73     | +                | +               | 4.7                            |
| H11Q   | 49     | 51     | +                | -               | 5.6                            |
| Q6R/H11N| 27     | 73     | +                | +               | 5.6                            |
| Q6R/H11T| 34     | 66     | +                | +               | 6.3                            |
| Q6R/H11Y| 60     | 40     | +                | -               | 5.5                            |
| L12F   | 28     | 72     | +                | +               | 8.1                            |
| L12S   | 91     | 9      | +                | +               | 2.8                            |
| L12V   | 12     | 88     | +                | +               | 6.9                            |
| L12W   | 55     | 45     | +                | +               | 6.1                            |
| L12M   | 11     | 89     | +                | +               | 8.8                            |
| L5R    | 91     | 9      | +                | +               | 6.3                            |

* ND = not detected.

tiang thiolases was determined using two assays. The first was a functional assay involving growth of *S. cerevisiae* on oleic acid-agar. The thiolase gene disruption strain STUD (Fig. 4, NULL) and the STUD strain carrying a plasmid expressing the gene coding for Δ1–16 thiolase (Fig. 4, Δ1–16) could not grow on oleic acid-agar, while the STUD strain carrying a plasmid expressing the wild-type thiolase gene (Fig. 4, WT) could grow on this medium. Therefore, growth on oleic acid-agar necessitates correct targeting of thiolase to peroxisomes. The second assay was biochemical, involving subcellular fractionation followed by immunodetection with anti-thiolase antibodies (Fig. 5).

The inability of the STUD strain expressing certain mutant thiolase genes to grow on oleic acid-agar was not due to the synthesis of enzymatically inactive thiolase in these transformants. All mutant thiolases were enzymatically active (Table I) at levels comparable to that of wild-type thiolase. The specific activities of mutant thiolases varied between 2.8 and 11.0 nmol min⁻¹ mg of protein⁻¹. The specific activity of wild-type thiolase was 6.6 nmol min⁻¹ mg of protein⁻¹. One mutant, L5R*, did not grow on oleic acid-agar (Fig. 4) and showed almost no thiolase activity (0.14 nmol min⁻¹ mg of protein⁻¹); however, this low activity was due not to the production of normal levels of a poorly active thiolase but to the low levels of thiolase synthesized (Fig. 5). A second isolate of this mutant, L5R, still showed reduced growth on oleic acid-agar (Fig. 4), even though it made increased amounts of thiolase (Fig. 5) that showed normal enzymatic activity (6.3 nmol min⁻¹ mg of protein⁻¹). No thiolase was detected either immunologically (Fig. 5, NULL) or enzymatically (Table I, NULL) in the STUD strain transformed with the parental vector pRS315.

Fig. 5. Immunodetection of thiolase in subcellular fractions of the parental and various thiolase-mutant strains of *S. cerevisiae*. All strains were grown in SCIM at 30 °C. For each strain, equivalent cellular fractions were run in the S (20 kgS) and P (20 kgP) lanes. Immunodetection of thiolase was by rabbit anti-thiolase serum followed by 125I-protein A. Strain designations are as in Fig. 4.
Only the mutant L5R and the double mutant L5V/Q6P showed retarded growth on oleic acid-agar at both 23°C and 30°C (Fig. 4) and in the almost total mislocalization of thiolase to the cytosol (Fig. 5). The single mutation Q6P resulted in a moderate reduction in targeting efficiency (Fig. 5). The Leu to Val substitution at position 5 in L5V/Q6P is conservative and might therefore be expected to result in little or no further diminution of targeting over that observed in Q6P alone. However, it appears that these side-by-side substitutions act synergistically to abolish targeting and import of thiolase into peroxisomes (Fig. 5), although we cannot formally rule out the possibility that the single mutant L5V would show the same characteristics as the L5V/Q6P double mutant. Indeed, for all double mutants, it is difficult to assess whether a single mutation in one or the other of the two amino acids or whether the two mutations acting in combination is responsible for the observed behavior of a particular double mutant thiolase. The L5R mutant examined initially was very poorly expressed (designated as L5R*) and was replaced by an alternative isolate expressing the same mutation at an acceptable level (Fig. 5). Neither L5R* nor L5R was effectively targeted, and neither could support growth on oleic acid-agar (Fig. 4).

The mutants R4S and L5Q supported growth on oleic acid-agar at 30°C (Fig. 4). These two mutants cultured at 30°C showed similar amounts of thiolase in their 20 kgP fractions as did mutants that showed poor growth on oleic acid-agar at 30°C (Figs. 4 and 5, compare R4S and L5Q with R4G, L12S, and L6R). This observation suggests that only small amounts of thiolase need be correctly targeted to peroxisomes to support growth on oleic acid-agar. The two mutants (R4G and L12S) targeted thiolase poorly (Fig. 5). These mutants grew poorly at 30°C but were able to grow better at 23°C on oleic acid-agar (Fig. 4). This temperature sensitivity may be due to thermal destabilization within the thiolase PTS, resulting in reduced interaction between it and factors involved in the recognition of the PTS.

Four mutations (S2A, R4A/L12M, L12V, and L12M) had a negligible effect on targeting (Fig. 5) and all showed reasonable growth on oleic acid-agar (Fig. 4). The conversion of Ser-2 to Ala (S2A) through the introduction of an negligible effect on targeting (Fig. 5). The Leu to Val substitution at position 5 in L5V/Q6P is conservative and might therefore be expected to result in little or no further diminution of targeting over that observed in Q6P alone. However, it appears that these side-by-side substitutions act synergistically to abolish targeting and import of thiolase into peroxisomes (Fig. 5), although we cannot formally rule out the possibility that the single mutant L5V would show the same characteristics as the L5V/Q6P double mutant. Indeed, for all double mutants, it is difficult to assess whether a single mutation in one or the other of the two amino acids or whether the two mutations acting in combination is responsible for the observed behavior of a particular double mutant thiolase. The L5R mutant examined initially was very poorly expressed (designated as L5R*) and was replaced by an alternative isolate expressing the same mutation at an acceptable level (Fig. 5). Neither L5R* nor L5R was effectively targeted, and neither could support growth on oleic acid-agar (Fig. 4).

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The double mutant R4A/L12M was efficiently targeted (Fig. 5) and supported strong growth on oleic acid-agar (Fig. 4). This result is difficult to explain given that the more conservative substitution R4K was less efficiently targeted and had decreased growth on oleic acid-agar. If the reduced volume of the Ala side chain compensated for the loss of the positively charged Arg side chain, then the mutant R4S should be expected to be accommodated as well as R4A. However, this was not the case, as the mutant R4S was poorly targeted. The possibility remains that the second amino acid mutation (L12M) in the double mutant compensates in an unknown way for the loss of Arg at position 4.

All thiolases characterized to date have a conserved Gln corresponding to position 6 in S. cerevisiae peroxisomal thiolase. Mutation of Gln at this position to Pre (Q6P) resulted in reduced targeting of the mutated thiolase vis-a-vis wild-type thiolase (Fig. 5). This reduced targeting might be the result of the loss of hydrogen bonding capacity at this position in the mutant. The mutant Q6H retains hydrogen bonding capacity and was more efficiently targeted than Q6P but still less well than wild-type thiolase. In contrast, thiolase in which the conserved His at position 11 is replaced by Gln (H11Q) was divided almost equally between the 20 kgP and 20 kgG. The mutant H11L was targeted more efficiently than H11Q.

Of the three double mutants encompassing Q6 and H11 (Q6R/H11N, Q6R/H11T, and Q6R/H11Y), Q6R/H11Y is the most interesting in that while ~40% of thiolase was localized to the 20 kgP in yeast grown at 30°C (Fig. 5), this mutant was incapable of growth on oleic acid-agar at 30°C (Fig. 4). Immunolocalization of thiolase to the 20 kgP cannot distinguish between thiolase correctly localized to peroxisomes and thiolase that is mislocalized to one or more compartments that co-sediment with peroxisomes in the 20 kgP. Density gradient centrifugation showed a preferential immunolocalization of the Q6R/ H11Y double mutant to fractions enriched for mitochondria (data not shown). Strong immunofluorescence from cytosolic thiolase did not permit an unequivocal localization of the Q6R/H11Y mutant by light microscopy (data not shown). The effect of mutating His-11 of S. cerevisiae thiolase may be similar to the effects seen by mutating the corresponding His residue (His-17) in the targeting signal of rat peroxisomal thiolase B. Mutation of His-17 of rat peroxisomal thiolase B to Arg, Lys, Leu, or Val resulted in the targeting of DHFR-PTS-2 fusion constructs to mitochondria and in mislocalization to the cytosol in CHO cells (Osumi et al., 1992). The change of Gln to Arg at position 6 in the double mutant Q6R/H11Y may exert an influence on the mistargeting of this mutant to mitochondria, for while the single mutants H11L and H11Q had more thiolase and a similar amount of thiolase, respectively, in their 20 kgP fractions than did Q6R/H11Y (Fig. 5), they still allowed for growth at both 23°C and 30°C on oleic acid-agar (Fig. 4). Therefore, in these two single mutants, some portion of the thiolase found in the 20 kgP fractions must be correctly sorted to peroxisomes so as to permit growth.

**DISCUSSION**

We have shown that the amino-terminal 16 amino acids of S. cerevisiae peroxisomal thiolase are both necessary and sufficient for protein targeting to peroxisomes *in vivo*. Therefore, S. cerevisiae peroxisomal thiolase has a PTS-2, akin to the PTS-2s of rat thiolases A and B. However, unlike the amino termini of peroxisomal thiolases from human and rat, the amino terminus of S. cerevisiae peroxisomal thiolase is not extensively cleaved upon import. In contrast, we have recently observed that peroxisomal thiolase is cleaved upon import into peroxisomes of the yeast *Y. lipolytica* (Nuttley et al., 1994). Therefore, the phenomenon of thiolase cleavage is conserved in at least one yeast species.

Mutations of amino acids within the PTS-2 of *S. cerevisiae* thiolase affect the localization and import of thiolase as indicated by subcellular fractionation and the ability to restore growth on oleic acid-agar to a thiolase-deficient strain. Although there is not always a tight correlation between increased amounts of thiolase in peroxisomes and increased growth on oleic acid-agar in the "spot" growth assay presented in Fig. 4, the results are consistent with a few conclusions. Substitution of Leu-5 by Arg completely abolishes import of thiolase by peroxisomes and retards growth on oleic acid-agar at both 23°C and 30°C. Substitution of Leu-5 by Gln compromises severely targeting to peroxisomes, causing most of the thiolase to be mislocalized to the cytosol. However, the small amount of thiolase imported into peroxisomes in the L5Q mutant permits reduced growth on oleic acid-agar. Similarly, when Leu-12 is replaced by hydrophobic amino acids with similar
volumes (Met and Val), there is little effect on targeting of thiolase to peroxisomes. However, when Leu-12 is replaced by much larger hydrophobic amino acids (Phe and Trp), thiolase targeting to peroxisomes is reduced. Substitution of a polar residue (Ser) at Leu-12 abolishes thiolase targeting to peroxisomes at 30 °C. The Leu residues at -23 and -16 of the PTS-2 of rat thiolase B correspond to Leu-5 and Leu-12 of S. cerevisiae thiolase. Helical wheel analysis showed that these Leu residues align in the two thiolases, suggesting that they form part of a hydrophilic surface required for interaction of the PTS-2s with their respective receptors. However, although our results suggest that small hydrophobic amino acids are required at positions 5 and 12, we have no direct evidence that the PTS-2 of S. cerevisiae thiolase forms a helical structure.

Arg-4 is a critical residue within the PTS-2 of S. cerevisiae thiolase. Substitution of Arg-4 by either Ser or Gly causes most of the thiolase to be mislocalized to the cytosol. Even the conservative substitution of Arg-4 by Lys results in significant mislocalization of thiolase to the cytosol, suggesting that the precise positioning of a positively charged group within the overall structure of the signal is required for maximum functioning of the targeting signal.

The existence of S. cerevisiae mutants that synthesize thiolases altered in their PTS and which are less efficiently targeted to peroxisomes than wild-type thiolase provides us with the means for isolating proteins that could interact with the thiolase PTS. We are currently exploring the possibility of suppressing the defect in peroxisomal import of certain mutant thiolases by overexpression of genes contained in a multicopy plasmid vector library. Such a strategy should allow us to identify genes whose products are involved in recognizing the thiolase PTS-2, thereby initiating import of thiolase into the peroxisomal matrix.

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