We have identified the \textit{Saccharomyces cerevisiae} gene \textit{ECI1} encoding \(\Delta^3\)-\(\Delta^2\)-\(\Delta^4\)-trans-enoyl-CoA isomerase that acts as an auxiliary enzyme in the \(\beta\)-oxidation of (poly)-unsaturated fatty acids. A mutant devoid of Eci1p was unable to grow on media containing unsaturated fatty acids such as oleic acid but was proficient for growth when a saturated fatty acid such as palmitic acid was the sole carbon source. Levels of \textit{ECI1} transcript were elevated in cells grown on oleic acid medium due to the presence in the \textit{ECI1} promoter of an oleate response element that bound the transcription factors Pip2p and P12061 (to B. H.), Jubila¨umsfonds der O¨sterreichischen National-
The ability of the null mutant to grow on various fatty acids. It was additionally used to examine the physiological role of rat peroxisomal MFE type I previously reported to contain $\Delta^3$-cis-$\Delta^2$-trans-enoyl-CoA isomerase activity in vitro (10). The results are discussed in terms of the function of conserved amino acid residues within the active site of members of the low homology hydratase/isomerase family of proteins.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The *S. cerevisiae* strains and plasmids used are listed in Table I. *Escherichia coli* strains DH10B, HB101, and DH5a were used for all plasmid amplifications and isolations. Yeast Media and Growth Conditions—For RNA isolations, logarithmic cultures of the strain BJ1991 were grown for 16 h in YP medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone) containing 2% (w/v) 2-glucose, 2% (w/v) ethanol, or 0.2% (w/v) oleic acid and 0.02% (w/v) Tween 80 (adjusted to pH 7.0 with NaOH) to an $A_{600}$ = 1.0. Plates used to assess utilization of fatty acids were prepared by pouring a thin layer of medium at 55 °C that consisted of 0.67% (w/v) yeast nitrogen base with amino acids, 0.1% (w/v) yeast extract, 0.5% (w/v) potassium phosphate at pH 6.0, and 2% (w/v) agar. The medium was autoclaved with 0.125% (w/v) palmitic acid (cis-3-C16:0), oleic acid, cis-12-octadecenoic acid, or arachidonic acid (cis-3-C20:4) and with 0.5% (w/v) Tween 80 to solubilize the fatty acid used. In oleic acid liquid medium used for the same purpose, the agar was omitted. Growth assays on solid media were performed by propagating cells overnight in liquid YPD (YP with 2% (w/v) 2-glucose) medium at 30 °C with shaking at 170 rpm. Following a further 5 h in fresh YPD, cells from logarithmic cultures were serially diluted, and aliquots of 2 μl were spotted on plates. For complementation of eci1Δ cells, transformants overexpressing Ecilp, rat peroxisomal MFE type I, or those harboring the vector were grown as above in liquid dropout media consisting of 0.67% yeast nitrogen base, 2% (w/v) 2-glucose, and amino acids (20 mg × 1 l) as required, and spotted on fatty acid plates. For enzyme assays, yAG526 and yAG627 cells from stationary phase cultures were grown for 18 h in medium containing oleic acid as described for RNA isolation that additionally contained 0.05% (w/v) 2-glucose and 75 μg × ml$^{-1}$ ampicillin. YPD-G418 plates used to select for yeast transformants carrying the kanMX4 deletion

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| **S. cerevisiae** | MATa leu2 ura3–52 trp1 pep4–3 prb1–1122 | Ref. 42 |
| 1) BJ1991 | MATa leu2 ura3–52 his4X lys HO::LYS | N. Klecknera |
| 2) NKY857 | eci1Δ | This study |
| 3) BJ1991eci1Δ | As above | This study |
| 4) NKY857eci1Δ | Corresponding wild type | Ref. 36 |
| 5) yAG760Δ | MATaΔ homologous eci1Δ disruptant | This study |
| 6) yAG162Δ | Corresponding wild type | Ref. 36 |
| 7) yAG141Δ | ssp1A::LEU2 | Ref. 36 |
| 8) yAG146Δ | ssp1A::LEU2 | Ref. 36 |
| 9) yAG161Δ | MATaΔ homologous ssp1AΔ disruptant | Ref. 36 |
| 10) BJ1991px6Δ | C16:0::LEU2 | Ref. 54 |
| yAG826Δ | pAG766 (YPEplac181::ECI1) overexpressing Ecilp | This study |
| yAG627Δ | YEp181 (vector) | This study |
| yAG653Δ | pYE352-CTA1 expressing Cta1p | This study |
| yAG856Δ | pYE352-rMFE expressing rat peroxisomal MFE type I | This study |
| yAG874Δ | pYE352-CTA1 expressing Cta1p | This study |
| BJ1991px1Δ | CTA1 Δ::LEU2 | Ref. 54 |
| BJ1991pip2Δsofa1Δ | CTA1 Δ::LEU2 | Ref. 26 |
| yAG872Δ | pADH2-GFP-ECI1 | This study |
| yAG873Δ | pADH2-GFP-ECI1 | This study |
| **Plasmid** | px6Δ::LEU2 | Stratagene |
| pSK::YLR284c | 1.5-kb ECI1 (H0390/2) in pSKX801 and Pestl | This study |
| pAG766 | 1.5-kb ECI1 (YLR284C-A1/A4) in YEp181/SmaI | This study |
| pAG832 | ECI1 ORF (YLR284C-ORE1/2) in pSK/SolI | This study |
| pBluescript SK(+) | Template for kanMX4-based PCR disruptions | Ref. 24 |
| pFA6a-kanMX | TRP1-containing integrative vector | Ref. 25 |
| YEp181 | LEU2-containing multicycopy vector | Ref. 25 |
| pYe352-rMFE | URA3-based multicopy vector harboring rMFE | Ref. 51 |
| pYe352-CTA1 | URA3-based multicopy vector harboring cta1 | Ref. 51 |
| pGEM::ECI1 | Containing a NotI- HindIII-delimited ECI1 | This study |
| pGEM::ECI1 | Containing a NotI- HindIII-delimited ECI1 | This study |
| pADH2-OAFl | Containing a NotI site; YEp184-based plasmid | Promega |
| pADH2-NOTT-ECI1 | NotI-preceded ECI1 for tagging | Ref. 26 |
| pADH2-GFP-ECI1 | GFP-Ecllp fusion linked to the ADH2 promoter | This study |
| pUC18::ECI1 | Containing PCR product using YLR-FWD/REV | This study |
| pUC18 | SureClone™ ligation kit | Amersham Pharmacia Biotech |
| pET3a::ECI1 | NdeI-BamHI ECI1 fragment of pUC18::ECI1 | This study |
| pET3a | Expression vector containing T7 promoter | Novagen |

a Harvard University.

b The numbers in superscript following the strains’ designations refer to their parental genotypes, e.g., BJ1991eci1Δ was derived from 1) BJ1991.
H0392 (YLR284C 3’-end) 5'-GAGTATACACTCAGGAGATTG-3' This study
H0392 (YLR284C 3’-end) 5'-GCTCGACCTAGAGATCCGATAG-3' This study
YLR-FWD 5'-CAGAATTCGATGTTGAC-3' This study
YLR-BACK 5'-GCGGCCGCAGAAGCTTCTAGA-3' This study
S1 (‘5’ of kanMX4) 5'-GTCGACGGTTCACCTTGGAGTGAGC-3' Ref. 24
S2 (‘3’ of kanMX4) 5'-GTCGACGCAGATTTCACCTGGAGTGAGC-3' Ref. 24
YLR284C-S1 5'-GCTCGACCTAGAGATCCGATAG-3' This study
YLR284C-S2 5'-GCTCGACCTAGAGATCCGATAG-3' This study
YLR284C-S1 5'-TTCGACCCGATGAGATCCGATAG-3' This study
YLR284C-S2 5'-TTCGACCCGATGAGATCCGATAG-3' This study
YLR284C-S3 5'-TTCGACCCGATGAGATCCGATAG-3' This study
YLR284C-S4 5'-TTCGACCCGATGAGATCCGATAG-3' This study
NOR1 5'-TTCGACCCGATGAGATCCGATAG-3' This study
NOR2 5'-TTCGACCCGATGAGATCCGATAG-3' This study
NOR3 5'-TTCGACCCGATGAGATCCGATAG-3' This study
NOR4 5'-TTCGACCCGATGAGATCCGATAG-3' This study
NOR5 5'-TTCGACCCGATGAGATCCGATAG-3' This study
NOR6 5'-TTCGACCCGATGAGATCCGATAG-3' This study
YOR284C 5'-TTCGACCCGATGAGATCCGATAG-3' This study
YOR284C 5'-TTCGACCCGATGAGATCCGATAG-3' This study

| Oligonucleotide | Description | Source or reference |
|-----------------|-------------|---------------------|
| H0392 (YLR284C 5’-end) 5'-GGTATTACACTTCAGGATGAGCGCCT-3' | This study |
| H0392 (YLR284C 3’-end) 5'-GGTATTACACTTCAGGATGAGCGCCT-3' | This study |
| YLR-FWD 5'-CAACATATCCCAACAAATATT-3' | This study |
| YLR-BACK 5'-GCGGCCGCAAGGCGGATCC-3' | This study |
| S1 (‘5’ of kanMX4) 5'-GTCGACGGGATCCCTTGGAGTGAGC-3' | Ref. 24 |
| S2 (‘3’ of kanMX4) 5'-GTCGACGCAGATTTCACCTGGAGTGAGC-3' | Ref. 24 |
| YLR284C-S1 5'-GCTCGACCTAGAGATCCGATAG-3' | This study |
| YLR284C-S2 5'-GCTCGACCTAGAGATCCGATAG-3' | This study |
| YLR284C-S1 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| YLR284C-S2 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| YLR284C-S3 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| YLR284C-S4 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| NOR1 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| NOR2 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| NOR3 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| NOR4 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| NOR5 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| NOR6 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| YOR284C 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |
| YOR284C 5'-TTCGACCCGATGAGATCCGATAG-3' | This study |

* S1 and S2 extend from the 3’-ends of the respective oligonucleotides that include them in their designation, e.g. S1 extends from YLR284C-S1.

Heterologous expression of Eci1p was performed by cloning pET3a::ECI1 into the plasmid pET3a::ECI1, resulting in pET3a::ECI1 transformed into BL21(DE3)pLysS (10 mM sodium phosphate, 2 mM potassium phosphate, 140 mM NaCl, 3 mM KCl, 5 mM 2-mercaptoethanol, pH 7.4), and plated in 15 ml of lysis buffer (20 mM potassium phosphate buffer at pH 7.6 and 100 mM KCl), and stored at −70 °C until needed. Following thawing, the lysed cells were incubated with RNase (2 μg/ml), DNase (20 μg/ml), and lysozyme (100 μg/ml) in the presence of 0.1 mM phenylmethylsulfonyl fluoride at 35 °C for 30 min. EDTA (3 mM) was added to the cell lysate, and cell debris was removed by centrifugation at 30,000 × g for 45 min at 4 °C.

Plasmid Constructions—All oligonucleotides used for constructing plasmids and verifying knockout strains are listed in Table II. The plasmid pSK:YLR284c containing ECI1 was constructed by inserting the XbaI- and PstI-digested polymerase chain reaction (PCR) product generated with oligonucleotides H0392 and H0392 using genomic yeast DNA as template into a similarly digested pBluescript SK+ vector (Stratagene, La Jolla, CA). The yeast shuttle vector overexpressing ECI1 (pAG766) was generated by ligating the amplified product of oligonucleotides YLR284C-A1 and YLR284C-A4 to a Smal-digested YEpplac181 (25). For electrophoretic mobility shift assays (EMSAs), the annealed SalI-digested oligonucleotides defining the ORF in the respective plasmids were digested with SalI and dephosphorylated, and a sample containing 30 mM KCl, 1 mM EDTA, and 0.5 mM benzamide hydrochloride. The column was washed with 120 ml of the same buffer, and bound Δ3-cis-Δ2-trans-enoyl-CoA isomerase activity was eluted with a 50-ml linear gradient of 0–0.25 M potassium chloride. Following their adjustment to pH 7.2, the pooled Δ3-cis-Δ2-trans-enoyl-CoA isomerase fractions containing isomerase activity were applied to an 1 ml of a modified, ice-cold, glass bead disruption buffer (28) that contained 0.1 mM benzamide hydrochloride, 0.1 mM EGTA, and 300 μM of acid-washed glass beads. Cell debris was removed by centrifugation for 10 min. The assay for Δ3-cis-Δ2-trans-enoyl-CoA isomerase was conducted according to published methods (10) using 60 μM trans-3-hexenoyl-CoA as substrate. The reaction was monitored by following the generation of a

Enzyme Assays—Soluble protein extracts were prepared by breaking oleic acid-induced cells (approximately 100–400 mg, wet weight) in 400 μl of a modified, ice-cold, glass bead disruption buffer. After centrifugation of the column with 5 ml of the same buffer, the isomerase activity was eluted with a 25-ml linear gradient of 0–0.35 M KCl. The purification of Eci1p was completed by applying a sample containing 0.92 μmol of isomerase activity to a Superdex 200 HR 10/30 column (Pharmacia Biotech) in equilibrium with a buffer consisting of 200 mM potassium phosphate (pH 7.2) and 3 mM EDTA.

Disruption of ECI1—Construction of ECI1-disrupted strains was performed according to the European Functional Analysis Network guidelines for the short flanking homology method, based on published protocols (24). The product obtained following PCR performed on pFA6a-kanMX4 template DNA using the 58- and 59-mer oligonucleotides YLR284C-A1 and YLR284C-A2 was used to transform S. cerevisiae strains BJ1991 and NRY857. Genomic-resident transformants that grew on YPD-G418 plates were verified for correct integration of the eci1:kanMX4 disruption fragment by PCR performed on genomic DNA using the oligonucleotide pairs YLR284C-A1/K2 and K3/YLR284C-A4 (24).
The ECI1 Gene Encodes \( \Delta^3 \)-cis-\( \Delta^2 \)-trans-Enoyl-CoA Isomerase

---

**RESULTS**

Identification of a Novel *S. cerevisiae* Open Reading Frame Encoding a Protein with Similarities to Other \( \beta \)-Oxidation Enzymes—A search of the data bases for novel genes that contained in their promoters sequences resembling OREs with the consensus CGGN_{15-18}CCG (22, 23) and that ended with nucleotides similar to those coding for a peroxisomal targeting signal type 1 (Ref. 41) revealed a homologous pair of open reading frames *YLR284c* (Fig. 2) and *YOR180c* that shared a moderate degree of amino acid sequence similarity to other hydratase/isomerase proteins. In the course of this work, the two genes appeared in searches conducted by other groups following a similar strategy and were reserved in the *Saccharomyces* Genome Data Base under designations reflecting homology to known enoyl-CoA hydratases type 1. As a consequence of findings presented here, *YLR284c* was named *ECI1*. *YOR180c* is the subject of current investigation and is not addressed further in this study.

**ECI1** Is Inducible under Oleic Acid Medium Conditions via

---

\(^a\)I. V. Karpichev, J. Lopez, and G. M. Small, manuscript in preparation.
an ORE—S. cerevisiae genes regulated by OREs are repressed in rich glucose medium, derepressed in media containing non-fermentable carbon sources such as ethanol, and are induced by the ORE-binding transcription factors Pip2p and Oaf1p (29, 43) when cells are grown in a medium containing oleic acid (20, 29). A DNA fragment consisting of ECI1 was hybridized with immobilized RNA that was obtained from cells grown in medium conditions supporting either repression, derepression, or induction (Fig. 3A). A strong signal was detected in the lane containing RNA from cells grown in oleic acid, and hence, the transcriptional profile of ECI1 was similar to that of the oleic acid-inducible genes POX1, encoding acyl-CoA oxidase (2), and SPS19, the yeast 2,4-dienoyl-CoA reductase (36). The observation that, like the transcripts of POX1 and SPS19, those of ECI1 did not accumulate in a strain devoid of Oaf1p and Pip2p indicated that up-regulation of ECI1 may be directed by the

ORE-like sequence identified in the data base search. To determine whether this element is bound by Oaf1p and Pip2p, an EMSA was performed.

The putative ECI1 ORE was assayed for possible protein-DNA interactions with Pip2p and Oaf1p using crude extracts from wild-type, pip2Δ, and oaf1Δ yeast cells propagated in oleic acid medium (Fig. 3B). The retarded complex formed with ECI1 ORE (indicated by an arrow) using protein extracts from wild-type cells was absent from the pip2Δ or oaf1Δ strains (26, 29). In addition to self competition, the signal of the retarded complex was reduced by excess unlabeled oligonucleotides containing the OREs of SPS19, POX1, and CTAl but not by a mutant version of the ORE of the latter gene (CTAlmut; Ref. 29). Lower and higher mobility complexes appearing in the vicinity of the indicated one were competed by ECI1 ORE and, to varying degrees, by the remaining OREs. However, since they also appeared in the lanes containing extracts from the pip2Δ or oaf1Δ mutants, their significance is not clear. From the Northern and EMSA analyses, it is concluded that the induction of ECI1 under oleic acid medium conditions was mediated by Pip2p and Oaf1p interacting with the ORE in the gene’s promoter. The fatty acid induction of ECI1 due to this ORE indicated that Eci1p may be involved in the breakdown of fatty acids. This could be examined by constructing a null mutant and determining its growth on a range of carbon sources.  

An eci1Δ Yeast Strain Is Defective for Growth on Unsaturated Fatty Acids—According to current understanding (Fig. 1), Δ3-cis-Δ5-trans-enoyl-CoA isomerase is required for degrading fatty acids with double bonds at both odd- and even-numbered positions in the carbon chain but is dispensable for breaking down saturated fatty acids. To elucidate the potential role of Eci1p in the metabolism of unsaturated fatty acids, wild-type and eci1Δ strains were grown on plates containing palmitic, cis-12-octadecenoic, oleic, or arachidonic acid as the sole carbon source. In these solid media, an emulsion is formed by adding Tween 80, which also acts as a poor carbon source, and therefore cells could grow on these plates, but zones of clearing indicate utilization of the additional fatty acid substrate. A pox1Δ strain was added to the plates to demonstrate lack of clear-zone formation on the fatty acids tested, and an sps19Δ disruptant acted as a control for utilization of unsaturated fatty acids with even-numbered double bonds (cis-12-octadecenoic acid and arachidonic acid with the cis-double bond at the Δ9-position). The eci1Δ-deleted strain was able to form a clearing zone when grown on palmitic acid but its growth was impaired on all three unsaturated fatty acids (Fig. 4). The
FIG. 5. Eci1p contains Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase activity. A, demonstration of Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase activity for Eci1p. Crude extracts from oleic acid-induced strains were reacted with 60 μM trans-5-hexenoyl-CoA as described under “Experimental Procedures.” The addition of protein from eci1Δ strain yAG827 harboring the YEpplac181 vector (arrow 1) did not result in the formation of a Mg\(^{2+}\) 3-ketohexanoyl-CoA complex; however, protein from yAG826 overexpressing Eci1p from plasmid pAG766 (arrow 2) yielded a specific Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase activity. B, Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase activity encoded by pAG766 was sufficient to restore the growth of the eci1Δ strain to wild-type levels on arachidonic acid, as demonstrated by the formation of a clearing zone in the medium. The wild-type and mutant strains containing the plasmid-borne ECI1 gene or the vector were yAG162, yAG826 (eci1Δ [ECI1]), and yAG827 (eci1Δ [Vector]), respectively.

FIG. 6. Pure bacterially expressed Eci1p was obtained following size exclusion chromatography. A, Superdex 200 HR 10/30 chromatogram of purified protein from the pooled Resource S fractions. B, Coomassie-stained SDS-polyacrylamide gel of the Superdex fractions. The numbers at the bottom of the lanes correspond to the fraction numbers in A. C represents 7.5 μg of protein combined from the previous four fractions.

ECI1 Encodes Δ\(^3\)-cis-Δ\(^2\)-trans-Enoyl-CoA Isomerase—Extracts from oleic acid-induced wild-type cells were examined for isomerase activity; however, this was below the detection limit of the assay used (<1 nmol × min\(^{-1}\) × ml\(^{-1}\) sample). Eci1p was therefore overexpressed in the eci1Δ mutant background from the multicopy plasmid pAG766 that contains the ECI1 gene under the control of the native promoter (yAG826), and this resulted in an isomerase activity of 60 nmol × min\(^{-1}\) × ml\(^{-1}\) sample). Furthermore, if Eci1p was incubated with trans-3-hexenoyl-CoA, NAD\(^{+}\), and 1,3-hydroxyacyl-CoA dehydrogenase, no reaction was observed until 2-enoyl-CoA hydratase 1 was added to the reaction mixture. These data indicated that the Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase (Eci1p) did not contain an additional detectable level of hydratase 1 activity. Also, no Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase activity (44) was observed when Eci1p was incubated with trans-3,5-hexadienoyl-CoA as substrate.

Eci1p Is Located in Peroxisomes—Eci1p does not contain an obvious N-terminal peroxisomal targeting signal type 2 sequence (45) but instead terminates with a peroxisomal targeting signal type 1-like tripeptide HRL (41, 46) that does not fully conform to the consensus sequence, (S/A/C)(K/H/R)(L/M). Hence, the protein’s location in cells was examined by tagging it with a fluorescent moiety. GFP has been amply used before for demonstrating the subcellular location of proteins and for marking peroxisomes (47, 48), and therefore an N-terminal GFP fusion with Eci1p was expressed from the ADH2 promoter. The GFP moiety of the fusion protein was monitored using fluorescent microscopy in S. cerevisiae wild-type and pex6Δ cells (49), which lack detectable peroxisomes that were grown under ethanol-medium conditions supporting ADH2 ac-

sequence. Eci1p eluted from a Superdex column at the same volume as rat Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase, which is a hexameric hydratase/isomerase protein with a native molecular mass of 170,000 Da (44). This value agrees with that obtained by dynamic light scattering analysis, which yielded only one signal peak that corresponded to an approximate molecular mass of 151,000 Da. In the final preparation, an overall purification of about 30-fold was obtained with a specific activity of 11.2 μmol × min\(^{-1}\) × mg\(^{-1}\) protein (Table III). The isomerase had a K\(_{m}\) value of 21.5 μM for trans-3-hexenoyl-CoA.

Purification and Characterization of Eci1p—To demonstrate that the yeast Δ\(^3\)-cis-Δ\(^2\)-trans-enoyl-CoA isomerase activity was chromatographically purified using DEAE-Sepharose (anion exchanger), Resource S (cation exchanger), and Superdex 200 HR 10/30 (size exclusion) columns (Fig. 6A). Analysis of SDS-polyacrylamide gels following electrophoresis revealed a single protein band with an apparent molecular mass of 32,000 Da (Fig. 6B), and this indicated that the protein was purified to apparent homogeneity. The polypeptide size obtained in this way agreed with the molecular mass of 31,700 Da calculated from the deduced amino acid
The ECI1 Gene Encodes Δ3-cis-Δ2-trans-Enoyl-CoA Isomerase

The purification protocol is described under "Experimental Procedures."

| Step                  | Protein | Total activity | Specific activity | Yield | Purification |
|-----------------------|---------|----------------|-------------------|-------|--------------|
| 30,000 × g supernatant | 87.8    | 30.9           | 0.35              | 100   | 1            |
| DEAE-Sephadex         | 5.0     | 11.2           | 2.24              | 38.2  | 6.4          |
| Resource S            | 1.56    | 10.6           | 6.79              | 34.3  | 19.4         |
| Superdex 200 HR 10/30* | 0.05   | 0.56           | 11.2              | 32    |              |

* Aliquot from Resource S containing 0.92 μmol × min⁻¹ Δ3-cis-Δ2-trans-eneoyl-CoA isomerase activity for Superdex 200 HR 10/30 chromatography.

**FIG. 7.** Eci1p is located in peroxisomes. The BJ1991 wild type and the otherwise isogenic pex6Δ strain expressing a GFP-Eci1p were propagated in YP medium containing 2% ethanol, and formaldehyde-fixed cells were examined for GFP fluorescence. Nuclei and mitochondria were detected by staining cells with 4,6-diamidino-2-phenylindole (DAPI). Nomarski images of the cells demonstrated their structural integrity.

**FIG. 8.** Rat peroxisomal MFE type 1 restores growth of the eci1Δ strain on oleic acid plates. Mutant eci1Δ cells expressing peroxisomal MFE type 1 (eci1Δ [rMFE]; yAG856) or catalase A (eci1Δ [Cta1p]; yAG874) were streaked on solid oleic acid plates, although in liquid medium it was less efficient at utilizing this carbon source (21).

All of the known *S. cerevisiae* genes encoding peroxisomally targeted β-oxidation enzymes, including POX1, FOX2, POT1/FOX3, and SPS19, are regulated via OREs (2–5, 22, 23, 36, 54). ECI1 was identified through a primary data base search for novel genes that could be inducible by oleic acid. The transcriptional profile of ECI1 was shown to be similar to that of other ORE-containing genes, and this depended on the presence of Pip2p and Ouf1p. The complete ECI1 ORE was found to contain one half-site conforming to the consensus 5'-CG-GNNNTNA-3' (22, 23) that bound the transcription factors...
The ECI1 Gene Encodes Δ3-cis-Δ2-trans-Enoyl-CoA Isomerase

Oaf1p and Pip2p (26, 29, 43, 55). The genomic organization of the ECI1 (YLR284c) locus resembles that of the ORE-containing SPS1/19 pair (36), since the divergent gene YLR285w is not up-regulated in oleic acid medium (data not shown). Although a comprehensive analysis of the ECI1/YLR285w intergenic region has not yet been completed, it would be reasonable to assume that there occurs an overlap of the ECI1 ORF by elements important for the regulation of YLR285w. Such elements could give rise to the complex protein-DNA interaction pattern seen in Fig. 5.

The deduced amino acid sequence of Eci1p is shown here to be moderately homologous (25–27% identity) to other members of the hydratase/isomerase family (56). One common structural feature of the hydratase/isomerase proteins revealed by x-ray crystallography (2-enoyl-CoA hydratase 1, 4-chlorobenzoyl-CoA dehalogenase, and Δ3,5-Δ2,4-dienoyl-CoA isomerase) is that for substrate binding and catalysis their subunits have a spiral core domain composed of four repetitive right-handed turns each consisting of two β-strands and an α-helix (39, 57, 58). The predicted secondary structure elements of Eci1p are highly conserved when compared with those of 2-enoyl-CoA hydratase 1 (Fig. 2). This indicates that the subunits of Eci1p may contain core domains (amino acid residues 20–169) that are similar to those of the other members of the hydratase/isomerase protein family. The predicted helical structures that correspond to trimerization domains 1 (amino acid residues 171–179 and 186–191 in Eci1p) and 2 (residues 225–237 and 244–252), and the connective loop (residues 204–218; Ref. 39) are also conserved, indicating that Eci1p could form a trimer. In line with this idea, size exclusion chromatography and dynamic light scattering gave for Eci1p a native molecular mass of 170,000 Da and 151,000 Da, respectively. When taking into account the polypeptide size (32,000 Da), this indicates that in the native state Eci1p is an oligomer, which we propose is a hexamer.

Amino acid sequence alignments of previously characterized hydratases and isomerases have shown that glutamate at the position equivalent to 164 in 2-enoyl-CoA hydratase 1 is conserved in all of them (18). Furthermore, site-directed mutagenesis studies have indicated that this glutamate participates in catalysis in both hydratase and isomerase reactions by transferring the proton at the C-2 carbon of the substrates (56, 59). Crystallographic studies of 2-enoyl-CoA hydratase 1 subsequently confirmed that Glu-164 is in the active site, acting as a catalytic acid in the hydration reaction (39, 40). Surprisingly, the amino acid sequence alignment revealed that Glu-164 of 2-enoyl-CoA hydratase 1 was replaced by Phe-150 in Eci1p (Fig. 2, arrow). This phenylalanine was confirmed by sequencing the nucleotides of the plasmid-borne gene used to express Eci1p in the immediate vicinity of substrate atoms that are susceptible to catalysis. Therefore, this tyrosine could potentially act as a catalytic acid residue.

Another insight gained from comparing Eci1p with hydratase/isomerase proteins is the importance of various amino acid residues in predicting whether members of this family are either hydratases or isomerases. Leu-130 of Eci1p is conserved in human and rat monofunctional mitochondrial isomerases (Fig. 2) and also in rat peroxisomal MFE type I (50). On the other hand, the corresponding amino acid residue in rat 2-enoyl-CoA hydratase 1 is Glu-144, which, according to x-ray crystallographic data, could act as the prerequisite catalytic base in the hydratase reaction (39, 40). It is also worth noting that the $K_m$ value of Eci1p is in the micromolar range similar to isomerases from bovine liver (32 μM) and from rat liver (37 μM; Refs. 14 and 56). The $V_{max}$ value of 11.2 μmol × min$^{-1}$ × mg$^{-1}$ protein is in the order of magnitude of that determined for rat mitochondrial monofunctional Δ3-cis-Δ2-trans-enoyl-CoA isomerase (35–40 μmol × min$^{-1}$ × mg$^{-1}$ protein; Ref. 10).

Thus, despite the low amino acid sequence similarity, the kinetic properties of Eci1p are comparable with those of its monofunctional mammalian counterparts.

The yeast Δ3-cis-Δ2-trans-enoyl-CoA isomerase mutant generated here represents the first organism devoid of this activity and has served to underpin the exact point of entry into β-oxidation of unsaturated fatty acids in a eukaryote. It confirmed the previous observation that the end product of yeast 2,4-dienoyl-CoA reductase (SpS1p), like that of its higher eukaryote counterpart, is 3-enoyl-CoA, since ECI1-deleted cells could not convert it to trans-2-enoyl-CoA (the substrate for Fox2p) and, therefore, could not grow on cis-12-octadecenoic acid. Finally, the eci1Δ strain provided proof that the Δ3-cis-Δ2-trans-enoyl-CoA isomerase activity contained in rat peroxisomal MFE type I was functional in vivo, since it was demonstrated that growth on unsaturated fatty acids could be restored using this heterologous enzyme.

Acknowledgments—We thank Hannelore Weba, Tanja Kokko, and Ville Ratasa for excellent technical assistance and Christian Schüller and Wolfram Görner for advice on the fluorescence microscopy and GFP work.

REFERENCES

1. Kunau, W. H., Buhne, S., Moreno de la Garza, M., Kionko, C., Mateblowski, M., Schulz-Borchard, U., and Thieringer, R. (1988) Biochem. Soc. Trans. 16, 418–420
2. Dmochowska, A., Dignard, D., Maleaska, R., and Thomas, D. Y. (1990) Gene (Amst.) 88, 247–252
3. Hiltunen, J. K., Wenzel, B., Beyer, A., Erdmann, R., Fossa, A., and Kunau, W. H. (1992) J. Biol. Chem. 267, 6646–6653
4. Igual, J. C., Matallan, E., Gonzalez-Bosch, C., Franco, L., and Perez-Ortin, J. E. (1991) Yeast 7, 379–371
5. Einerhand, A. W. C., Voom-Brouwer, T. M., Erdmann, R., Kunau, W. H., and Tabak, H. F. (1991) Eur. J. Biochem. 206, 113–122
6. Schultz, H., and Kunau, W. H. (1993) Trends Biochem. Sci. 12, 403–406
7. Hiltunen, J. K., Filipušula S. A., Koivuranta, K. T., Siivari, K., Qin, Y.-M., and Hayrinen, H. M. (1996) Ann. N. Y. Acad. Sci. 804, 116–128
8. Stoffel, W., and Grol, M. (1978) Hoppe-Seyler’s Physiol. Chem. 359, 1773–1792
9. Kilponen, J. M., Palosaari P. M., and Hiltunen, J. K. (1990) Biochem. J. 269, 223–226
10. Palosaari P. M., and Hiltunen, J. K. (1990) J. Biol. Chem. 265, 2446–2449
11. Kilponen, J. M., and Hiltunen, J. K. (1993) FEBS Lett. 322, 299–303
12. Kilponen, J. M., Hayrinen, H. M., Rehn, M., and Hiltunen, J. K. (1994) Biochem. J. 300, 1–5
13. Janssen, U., Fink, T., Lichten, P., and Stoffel, W. (1994) Genomics 23, 223–228
14. Euler-Bertram, S., and Stoffel, W. (1990) Biol. Chem. Hoppe-Seyler 371, 603–610
15. Struijk, C. B., and Beerthuis, R. K. (1966) Biochim. Biophys. Acta 116, 12–22
16. Engeland, R., and Kindl, H. (1991) Eur. J. Biochem. 196, 699–705
17. Preissig-Müller, R., Gühnemann-Schäfer, K., and Kindl, H. (1994) J. Biol. Chem. 269, 20475–20481
18. Wu, W.-J., Anderson, V. E., Raleigh, D. P., and Tonge, P. J. (1997) Biochemistry 36, 2211–2220
19. Stoffel, W., Diker, M., and Hofmann, K. (1993) FEBS Lett. 322, 119–122
20. Veenhuis, M., Mateblowski, M., Kunau, W. H., and Harder, W. (1987) Yeast 3, 77–84
21. Henke, B., Girzalski, W., Bertaux-Lecellier, V., and Erdmann, R. (1998) J. Biol. Chem. 273, 3768–3773
22. Filipsits, M., Simon, M., Matz, W., and Ruis, H. (1993) Gene (Amst.) 132, 49–55
23. Einerhand, A. W. C., Kos, W., Distel, B., and Tabak, H. F. (1993) Eur. J. Biochem. 214, 323–331
24. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) Yeast 10, 1730–1808
25. Grietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534
26. Rottensteiner, H., Kal, A. J., Hamilton, B. R., Ruis, H., and Tabak, H. F. (1997) Eur. J. Biochem. 247, 776–783
27. Görner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer,
The ECI1 Gene Encodes Δ^3-cis-Δ^2-trans-Enoyl-CoA Isomerase