Candida tropicalis Expresses Two Mitochondrial 2-Enoyl Thioester Reductases That Are Able to Form Both Homodimers and Heterodimers

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Here we report on the cloning of a Candida tropicalis gene, ETR2, that is closely related to ETR1. Both genes encode enzymatically active 2-enoil thioester reductases involved in mitochondrial synthesis of fatty acids (fatty acid synthesis type II) and respiratory competence. The 5′- and 3′-flanking (coding) regions of ETR2 and ETR1 are about 90% (97%) identical, indicating that the genes have evolved via gene duplication. The gene products differ in three amino acid residues: Ile67 (Val), Ala92 (Thr), and Lys251 (Arg) in Etr2p (Etr1p). Quantitative PCR analysis and reverse transcriptase-PCR indicated that both genes were expressed about equally in fermenting and ETR1 predominantly respiring yeast cells. Like the situation with ETR1, expression of ETR2 in respiration-deficient Saccharomyces cerevisiae mutant cells devoid of Ybr026p/Etr1p was able to restore growth on glycerol. Triclosan that is used as an antibacterial agent against fatty acid synthesis type II 2-enoyl thioester reductases inhibited growth of Fah1 overexpressing mutant yeast cells but was not able to inhibit respiratory growth of the ETR2- or ETR1-complemented mutant yeast cells. Resolving of crystal structures obtained via Etr2p and Etr1p co-crystallization indicated that all possible dimer variants occur in the same asymmetric unit, suggesting that similar dimer formation also takes place in vivo.

Candida tropicalis is an asporogenic diploid yeast that is specialized for growth on lipid-rich media. When n-alkanes or fatty acids are available as a carbon and energy source, C. tropicalis cells enhance expression of several lipid-metabolizing enzymes (1, 2). In this organism, alternative transcripts of a single gene or copies of transcripts arising from closely related but separate genes occur frequently (3–5). Consequently, these enzymes are present as an array of isoforms, each of which can be targeted to different subcellular locations and may serve either identical or different metabolic functions. This genetic redundancy might be used as a back-up system to sustain central cellular needs (6, 7). For example, peroxisomal acyl-coenzyme A (CoA) oxidase (Pox) is a key enzyme of β-oxidation that is present as multiple forms in C. tropicalis (1, 8, 9). At least three POX genes are present in the C. tropicalis genome. The closely related POX4 and POX5 have been shown to be differentially expressed depending on what lipids are available for growth (1).

Recently, we reported on the identification of a novel mitochondrial 2-enoil thioester reductase Etr1p from C. tropicalis (10) and also demonstrated that a homologous Saccharomyces cerevisiae protein Ybr026p involved in mitochondrial respiratory function (11) exhibits the same activity. Disruption of the corresponding nuclear gene in S. cerevisiae results in a respiratory-deficient strain unable to grow on nonfermentable carbon sources (11). S. cerevisiae Etr1p was suggested to link the assembly of the respiratory complexes with prokaryotic type fatty acid synthesis (type II) in fungal mitochondria (10, 12–16).

In the present study we describe a further C. tropicalis mitochondrial 2-enoil thioester reductase, Etr2p, that is closely related to Etr1p and compare the function of the two enzymes. Quantitative PCR was used to determine the expression pattern of the two reductase genes in C. tropicalis. The ability of ETR2 to rescue the respiratory-deficient phenotype of the ybr026cΔ strain from S. cerevisiae was also studied. Crystallization experiments on a mixture of purified Etr2p and Etr1p was undertaken to see whether homo- and heterodimeric variants were possible. The results are discussed in terms of genetic redundancy in lipid metabolism and its significance for the maintaining mitochondrial respiratory function.

Experimental Procedures
Cloning of ETR2 and ETR1—The primers used in this study are described in Table I. The CTGEN1 genomic fragment revealing the sequence for ETR1 and other genomic fragments, among them CTPU25 and CTHAE10 (see Fig. 1A), were obtained by PCR, ligation-mediated PCR, and screening of a C. tropicalis genomic DNA library as described (10). The sequences of the partially overlapping genomic fragments (CTPU25, CTS5PCR, CTPVUB10, and CTHAE10; see Fig.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U94996.

The atomic coordinates and structure factors (code IN96) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).}

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† The abbreviations used are: CoA, coenzyme A; ETR, 2-enoil thioester reductase; ORF, open reading frame; RST, random sequenced tag; RACE, rapid amplification of cDNA ends.

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41213
CTRED18
CTCGCCAGTTATCTGTTATCG

CTRED19
GAAGCCACCACACGCAACGTC

CTRED2R
GAGGGTTCGCCCAACTTCCACCA

Val67IleRev
CCAGATCTCCCTGGCTTCTCAATCTCA

Val67IleForw
CGAGGTCTCCTAGGGTCGCCCA

Val67IleRev
CGAGGTCTCCTAGGGTCGCCCA

Thr92AlaForw
CTGGTGTGGCCACGCGCACTTCG

Thr92AlaRev
GGCTGGTGTGGCCACGCGCACTTCG

Arg525LysForw
GACAGAAGACCTCAAAAATGGCGGCTTACCC

Arg525LysRev
GATTGGGCACTTTTCTGGCTGAATGGCTG

ETR1sense
GTCGCCGGCGTGAGCC

ETR1antisense
CAACCTTCTGGGCAAGCTATG

ETR2sense
TCACCACACGCAAGCCCG

ETR2antisense
TTACCGCAAGCGTTCTCAG

ETR1fluoro
Fam-CCTCCTACCTGCTGTTAAGACTCT-Tamra

ETR2fluoro
Fam-AAGACTACCTGCTGTTAAGACCT-Tamra

18Ssense
TGTCCTGCTGCTGAACTTGAAG

18Santisense
AGTCGAACTAATGAAAACG

18Sfluoro
Vic-CCTCCTACCTGCTGTTAAGACTCT-Tamra

The primers for ETR2 were highly similar but not identical in all regions. Primers CTRED18 and CTRED19 corresponding to the 5'-flanking and reverse 3'-flanking sequences of CTPYu25 and CTHeA1010, respectively, were used in PCR with genomic DNA as template. A DNA fragment of 3 kb was obtained (CTGEN2; see Fig. 1A), cloned into pUC18, and sequenced, revealing the sequence for ETR2.

**Pulsed Field Gel Electrophoresis—**Preparation of *C. tropicalis* chromosomal DNA and pulsed field gel electrophoresis were carried out according to the Bio-Rad instruction manual for CHEF-DR pulsed field electrophoresis system (Rev. B, Bulletin # 9086), available on the Bio-Rad Website (www.Bio-Rad.com) and the protocol for the preparation of agarose-embedded yeast DNA. The correct amount of cells required for the procedure was estimated by spinning down the cells to obtain a wet pellet corresponding to a volume of 50 μl.

**Preparation of cDNA**—Preparation of cDNA was carried out in linear mode. The mass spectra were calibrated with bovine serum albumin (Sigma-Aldrich). Mass measurements were carried out on an Applied Biosystems Voyager-DETM STR Biospectrometry work station operating in linear mode. The mass spectra were calibrated with bovine serum albumin as an external standard.

**Determination—**Crystals containing the dimers Etr1p-Etr1p, Etr1p-Etr2p, and Etr2p-Etr2p were obtained with the hanging drop method (22) using 20 mg/ml mixture of Etr1p:Etr2p in a 1:1 ratio and 1.8 M (NH4)2SO4, 0.1 M N-(2-acetamido)-2-iminodiacetic acid/NaOH (pH 7.0) as a precipitant. Crystallization drops were also supplemented with 5 mM NADPH and 1 mM octanoyl-CoA. The data for the crystal structure were collected at the beam line 1711 of the MAX-lab synchrotron (Lund, Sweden). The structure was solved with molecular replacement using the program AmoRe (CCP4 suite). Analysis of Etr1p apoenzymatic crystal structure without bound NADPH has been reported previously (23) and was used as a starting model in the calculations. Refinement of the structure was initiated by one cycle of simulated annealing using CNS (24) and continued with Refmac_5 (CCP4 suite) employing TLS (25). Water molecules were added with Arp/Warp (26) in cycles of 200 molecules followed by successive refinement runs and rebuilding in the program O (27). The ligand could be reliably built into the structure only after all waters were assigned at the final stage of the refinement, when only small gaps were present in the ligand density and with clear density for the phosphates and the ring structures. The crystal characteristics and refinement statistics are shown in Table II. The geometries of the structures were analyzed with the programs O, WHAT IF (28), and PROCHECK (29). All of the structure drawings were created with Swiss-PDBViewer (30) and edited with Adobe Photoshop 7.0.
able carbon sources, S. cerevisiae BJ1091, yhr082cΔ cells or those transformed with plasmid DNA for overexpression of Yhr026p, enoyl-ACP reductase (FabI) from E. coli, catalase (Cat1p), Etr2p, or Etr1p (10) were cultured on synthetic complete medium supplemented with 3% glycerol. Glycerol medium plus 0.125, 0.5, or 1.0 μg/ml of triclosan was prepared to examine the effect of the drug on the reductase function and viability of respiring yeast cells. After autoclaving the culture medium, triclosan was added from a 10 mg/ml (ethanol) stock solution. Triclosan was a gift from Louis Widmer (Dermatologica Widmer, Helsinki, Finland).

RESULTS

C. tropicalis Genomic DNA Contains a Novel Gene ETR2 Related to ETR1—Sequencing of a C. tropicalis genomic fragment (CTGEN2; see “Experimental Procedures”) revealed an open reading frame (ORF) of 1158 nucleotides, encoding a polypeptide of 386 amino acid residues that was termed Etr2p (Fig. 1A). CTGEN2 included 958 and 828 bp of the 5'- and 3'-flanking sequences from ETR2, respectively. The nucleotide sequences of the ETR2 and ETR1 (10) were compared and found to be 98.6% identical. Differences between Etr2p and Etr1p were seen in three residues: Ile67 (Val), Ala92 (Thr), and Lys251 (Arg) in Etr2p from glucose (oleic acid) grown yeast cells, the longest amplified fragments in each of the genomic DNA samples, which had hybridized to genomic DNA after Southern blot analysis of genomic DNA from C. tropicalis

Both ETR2 and ETR1 Are Expressed in C. tropicalis under Various Growth Conditions—5’-RACE was carried out with total RNAs of 2% glucose and 0.2% oleic acid-grown C. tropicalis cells. Twenty five (twenty) 5’-RACE clones were obtained from glucose (oleic acid) grown yeast cells, the longest amplified fragments extending to the ~33 (~19) position in the 5’-untranslated region. The 5’-RACE analysis showed that thirteen (six) amplified fragments presented ETR2 and twelve (fourteen) fragments presented ETR1 among the 5’-RACE clones from glucose (oleic acid)-grown yeast cells.

To quantitate ETR2 and ETR1 expression on different media quantitative PCR was carried out. Total RNA was extracted from cells grown on media containing glucose or oleic acid as sole carbon source. RNA was reverse transcribed to cDNA and amplified by PCR for quantification with ETR2- and ETR1-specific primers (see “Experimental Procedures”). Relative values for the amount of ETR2 RNA were 1.00 on glucose and 2.17 on oleic acid grown cells and for the amount of ETR1 RNA 1.31 on glucose and 2.95 on oleic acid, indicating that both genes were more strongly expressed on oleic acid than on glucose as a carbon source (Table III).

The ETR2 Gene Product Encodes an Etr1p-like 2-Enoyl Thioester Reductase with a Preference for Short and Medium Chain over Long Chain Enoyl Thioesters—to analyze the function of Etr2p as a 2-enoyl thioester reductase and to characterize its substrate specificity, it was overexpressed in S. cerevisiae and chromatographically purified to apparent homogeneity. As a comparison, Etr1p was also examined. When the purified proteins were analyzed by circular dichroism spectroscopy, the overall spectra of Etr2p and Etr1p were identical, signifying preservation of the secondary structure elements. Mass spectrometry analysis gave molecular masses which corresponded to theoretical values of 39.34 and 39.39 kDa for Etr2p and Etr1p, respectively, excluding the mitochondrial targeting signals, not present in the mature proteins (10). The mass difference was attributed to the difference in three amino acid residues deduced from cDNA sequence (Fig. 2). The specific reductase activities were determined using trans-2-hexenoyl-CoA (C6), trans-2-decenoyl-CoA (C10), and trans-2-hexadecenoyl-CoA (C16) as substrates. The respective values obtained for Etr2p and Etr1p were 17.3 and 18.0 μmol/min/mg of protein with trans-2-hexenoyl-CoA, 13.6 and 11.7 μmol/min/mg of protein with trans-2-decenoyl-CoA, and 4.3 and 5.8 μmol/min/mg of protein with trans-2-hexadecenoyl-CoA. Hence, both proteins showed a similar preference toward short and medium chain 2-enoyl thioesters. Gas chromatographic analysis of the reaction end products demonstrated that, like Etr1p (10), also Etr2p was able to carry out the reduction of the trans-2 double bond in monounsaturated trans-2- and conjugated trans-2,4-enoyl thioesters. Similarly to Etr1p, Etr2p was specific for NADPH and could not utilize NADH as hydrogen donor in the catalysis.

Etr2p-Etr1p Heterodimerization Enables Generation of Various Native Forms of the Enzyme—The present results indicated that C. tropicalis simultaneously expresses two highly similar enoyl thioester reductases. Because previous data using purified Etr1p indicated that it functions as a dimer (23), this raised the issue of whether the two reductases may form a heterodimer. Purified Etr2p and Etr1p were mixed, and crystals were grown that diffracted at 1.98 Å resolution. Determination of the crystal structure allowed the identification of the locations of Ile67 (Val), Ala92 (Thr), and Lys251 (Arg) in Etr2p (Etr1p). Detection of these different amino acids in the asymmetric unit in the crystal revealed the existence of all possible dimer variants, Etr1p-Etr1p, Etr1p-Etr2p, and Etr2p-Etr2p (Fig. 3, A and B). Fragmented electron density for the NADPH co-factor could be seen in three of six polypeptide chains in the asymmetric unit. Each type of dimer was liganded with one NADPH, and in the heterodimer Etr1p binds the co-factor in this crystal (Fig. 3A). Thus, the structure contains both Etr2p and Etr1p in liganded and unliganded form. Electron density for the octanoyl-CoA, present in the crystallization mixture, was not observed.

When comparing the apo-Etr1p component in the heterodimeric crystal to the structures of Etr1p with and without the bound ligand (Ref. 23; 1GUF and 1GU7 in the Protein Data Bank, respectively), it is noted that apo-structures show identical conformation and the conformational change from the apo- to holoenzyme form upon binding of NADPH (Fig. 3, C and D) is similar for the two proteins. It is also worth noting that despite this dynamics in the molecule, the apo- and holoenzymes are sufficiently similar as crystallizable species such that they can be incorporated into the same crystal lattice. The three amino acids distinguishing Etr2p and Etr1p were neither involved in crystal contacts nor restricted dimer formation. Of the differing amino acids Ala92 (Thr) and Lys251 (Arg) are located on the protein surface facing the solvent; the former in the middle of a large loop between helix α2 and strand β4 and the latter in the beginning of helix αE (the nomenclature of
ETR2 and ETR1 were obtained by ligation-mediated PCR. CTGEN2 was obtained by PCR amplification from genomic DNA. The positions of the oligonucleotides CTRED18 and CTRED19 are indicated by arrowheads, and ORFs are shown as boxes. CTGEN2 included an additional ORF (ORF2) downstream from ORF-ETR1, encoding a putative polypeptide of 163 amino acids with a similarity of 66% to the N-terminal 134 amino acids of allantoinase from Saccharomyces cerevisiae (accession number S48489). The triplets for initiation (ATG) and stop codons (TAG or TGA) are indicated. B, ETR genes as revealed by Southern blot analysis. Yeast genomic DNA (10 μg) was digested with EcoRI (1), HindIII (2), BamHI (3), EcoRI+HindIII (4), EcoRI+BamHI (5), or HindIII+BamHI (6) and loaded onto an agarose gel. After electrophoretic fractionation and blotting, the nitrocellulose filter was hybridized with 32P-labeled CTGEN2. The sizes (kb) of marker fragments are indicated on the left. C, chromosomal localization of ETR1 and ETR2 by pulsed field gel electrophoresis. C. tropicalis and Hansenula wingei (DNA size marker) chromosomal DNA was separated on 1.0% agarose gel (Fig. 4). Triclosan was not inhibitory for the growth of yeast cells on rich glucose (data not shown).

DISCUSSION

This work reports on the characterization of a novel gene ETR2, whose product functions as a mitochondrial 2-enoyl thioester reductase. In the process of cloning ETR1 (10), genomic fragments were isolated that contained an ORF (ETR2) very similar to that of ETR1. The ORFs were 96.8% identical at the nucleotide level, encoding proteins differing in three amino acid residues only. Differences in the 5' and 3'
regions of the two ORFs were more pronounced (90.2 and 89.2%, respectively). Purified Etr2p contained enoyl-CoA reductase activity, and expression of Etr2p in the respiration-deficient S. cerevisiae ybr026c/H9004 strain (11) was able to restore growth to the disruption strain on glycerol. These data demonstrated that C. tropicalis contained at least two functional 2-enoyl thioester reductases.

The phenomenon of genetic redundancy and the presence of different protein isoforms is widespread. In addition to being a fuel for evolvement of protein families, the presence of more than one functional copy of a gene can be seen as advantageous, if genes perform more than one specific function or are functioning under different conditions (6). Redundancy is common for all species and can be found even in the most simple genomes such as that of the parasitic Mycoplasma genitalium and extending to more complex multicellular eukaryotic genomes that may rely on redundant genes during development (6).

Although the random sequenced tags (RSTs) available at the Genolevures data base, the data bank of RSTs of a group of hemiascomycetous yeasts, at present cover only about 20% of the genome of C. tropicalis, genomic exploration of sequences as well as combining the data in the literature demonstrate growth to the disruption strain on glycerol. These data demonstrated that C. tropicalis contained at least two functional 2-enoyl thioester reductases.

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that a number of fatty acid metabolism (Table IV) enzymes encoded by single genes in *S. cerevisiae* have more than one copy of the corresponding genes in *C. tropicalis* (7, 40). The database search and the literature revealed that 3,5-2,4-dienoyl-CoA isomerases, 3-2-enoyl-CoA isomerases, multifunctional enzymes type-2, acetoacetyl-CoA thiolases, carnitine acetyltransferases, and acyl-CoA oxidase (1, 8, 41–43) together with Etr1p and Etr2p, belong to this category (Table III). Because the percentage of coverage of the *C. tropicalis* genome is still small and many of genes from *S. cerevisiae* wait for an identification of their orthologs in *C. tropicalis*, the number of genes in the group presenting genetic redundancy is likely to increase in the future.

Comparison of the amount of RNA between glucose- and oleic acid-grown cells indicated that both *ETR2* and *ETR1* were simultaneously present under both conditions. Both gene products were present in equal amounts in cells grown on glucose, but their expression levels are increased upon a shift of cells from fermentable (glucose) to nonfermentable (oleic acid) carbon source in a way that *ETR1* was the more predominantly expressed form. Aerobic organisms have evolved sensory systems to monitor oxygen availability, and, for instance in *S. cerevisiae*, there is a subset of genes of which transcription involves sensing oxygen and hypoxia (44). However, no changes were shown in the expression of *ETR2* and *ETR1* when cells were shifted to grow from respiration to anoxia (data not shown). The increased expression of both *ETR2* and *ETR1* on nonfermentable carbon source, oleic acid, suggests that the gene products are linked to cellular respiration.

**Fig. 3. Schematic representation of the Etr1p-Etr2p crystal structure.**

A, the contents of one asymmetric unit of the Etr1p-Etr2p crystal unit cell. Polypeptide chains of Etr1p are shown in blue, and Etr2p is in red (backbone atoms shown only). The NADPH co-factor is shown in black. The crystal is made up of the three possible dimeric protein combinations: Etr1p-Etr2p heterodimer (left), Etr1p-Etr1p homodimer (middle), and Etr2p-Etr2p homodimer (right). In each dimer only one monomer binds the co-factor.

B, stereo view of ribbon drawing of the structure of Etr2p with the residues differentiating it from Etr1p indicated. The Cα trace of the β-strands (blue), α-helices (red), and other polypeptide chains (gray) as well as NADPH are also shown. C, Etr1p with a bound NADPH superimposed on Etr2p with a bound NADPH. The conformation of polypeptide chains and co-factors are virtually identical (residual mean and standard deviation on all backbone atoms 0.81 Å). D, Etr2p with a bound NADPH (red) superimposed on Etr2p without NADPH (green). Only Cα atoms are shown. Conformational change induced by co-factor binding, as seen earlier for Etr1p (23) is apparent in the co-factor binding Rossmann fold domain (residual mean standard deviation on all backbone atoms 1.14 Å).

**Fig. 4. Effects of triclosan on the growth of the yeast cells.** The respiration-dependent growth of different yeast transformants was examined on glycerol with or without the addition of the drug triclosan (0.125, 0.5, and 1.0 μg/ml) in the growth medium. The strains examined are BJ1991 ybr026cΔ (1), BJ1991 ybr026cΔ transformed with pYE352::MRF1 (2), pYE352::ETR1 (3), pYE352::ETR2 (4), pYE352::FAB1 (5), or pYE352::CTA1 (6). The plates were incubated for 5 days at 30 °C.
architectures in the vicinity of the active sites in the both proteins were similar, and the amino acid residue differences Ile<sup>67</sup> (Val), Ala<sup>92</sup> (Thr), and Lys<sup>251</sup> (Arg) in Etr2p (Etr1p) were important in the maintenance of respiratory-competent mitochondria in yeast. Failure to inhibit the respiratory growth that depended on Etr1p, Etr2p, or Ybr026p suggested that triclosan does not inhibit reductases of the medium chain dehydrogenases/reductases protein family at the concentration effective against the short chain dehydrogenases/reductase type of bacterial enoyl thioester reductases.

Close similarity of <i>ETR2</i> and <i>ETR1</i> indicates the genes have arisen via gene duplication recently in the evolution. The higher identity of the nucleotide in the coding region than of in the 5′- and 3′-flanking regions of the genes indicates the importance of both functional and structural integrity of the proteins in <i>C. tropicalis</i>. The allowance of mutations at the promoter regions of genes is reflected by the different ratios of the <i>ETR2</i> and <i>ETR1</i> expression levels under fermentable and non-fermentable growth conditions. This suggests that at their promoter regions, binding of transcription controlling factors, which remain to be identify in future, have changed.

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