Mitochondrial trans-2-Enoyl-CoA Reductase of Wax Ester Fermentation from Euglena gracilis Defines a New Family of Enzymes Involved in Lipid Synthesis*

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Under anaerobiosis, Euglena gracilis mitochondria perform a malonyl-CoA independent synthesis of fatty acids leading to accumulation of wax esters, which serve as the sink for electrons stemming from glycolytic ATP synthesis and pyruvate oxidation. An important enzyme of this unusual pathway is trans-2-enoyl-CoA reductase (EC 1.3.1.144), which catalyzes reduction of enoyl-CoA to acyl-CoA. Trans-2-enoyl-CoA reductase from Euglena was purified 1700-fold to electrophoretic homogeneity and was active with NADH and NADPH as the electron donor. The active enzyme is a monomer with molecular mass of 44 kDa. The amino acid sequence of tryptic peptides determined by electrospray ionization mass spectrometry were used to clone the corresponding cDNA, which encoded a polypeptide that, when expressed in Escherichia coli and purified by affinity chromatography, possessed trans-2-enoyl-CoA reductase activity close to that of the enzyme purified from Euglena. Trans-2-enoyl-CoA reductase activity is present in mitochondria and the mRNA is expressed under aerobic and anaerobic conditions. Using NADH, the recombinant enzyme accepted crotonyl-CoA (k_m = 68 μM) and trans-2-hexenoyl-CoA (k_m = 91 μM). In the crotonyl-CoA-dependent reaction, both NADH (k_m = 109 μM) or NADPH (k_m = 119 μM) were accepted, with 2-3-fold higher specific activities for NADH relative to NADPH. Trans-2-enoyl-CoA reductase homologues were not found among other eukaryotes, but are present as hypothetical reading frames of unknown function in sequenced genomes of many proteobacteria and a few Gram-positive eubacteria, where they occasionally occur next to genes involved in fatty acid and polyketide biosynthesis. Trans-2-enoyl-CoA reductase assigns a biochemical activity, NAD(P)H-dependent acyl-CoA synthesis from enoyl-CoA, to one member of this gene family of previously unknown function.

The mitochondrion of the photosynthetic flagellate Euglena gracilis is a facultatively anaerobic organelle that produces ATP under aerobic and anaerobic conditions. In the presence of oxygen, pyruvate from glycolysis enters the mitochondrion and undergoes oxidative decarboxylation, the resulting acetyl-CoA enters a modified tricarboxylic acid cycle entailing a succinatesemialdehyde shunt as found in the α-proteobacterium Bradyrhizobium (1). Electrons from glucose breakdown are transferred to oxygen as the terminal electron acceptor (2, 3).

In the absence of oxygen, acetyl-CoA stemming from pyruvate serves as the terminal electron acceptor of glucose oxidation via an unusual mitochondrial fatty acid synthesis (4). The shift to anaerobic conditions leads to the malonyl-CoA-independent synthesis of wax esters, levels of which can reach up to 40 μg/10^6 Euglena cells, from its reserve polysaccharide paramylon (5). The wax ester fatty acids are synthesized from acetyl-CoA stemming from pyruvate via an unusual oxygen-sensitive enzyme, pyruvate:NADP^+ oxidoreductase (6, 7), the core catalytic component of which is pyruvate:ferredoxin oxidoreductase, a typical enzyme of hydrogenosomes (8). Pyruvate:NADP^+ oxidoreductase exists in the mitochondrion alongside a classical mitochondrial pyruvate dehydrogenase complex with mRNA expression patterns converse to that of pyruvate:NAD^+ oxidoreductase in response to hypoxia (9).

Five different systems of fatty acid synthesis have been reported for E. gracilis (3). The first two are acyl carrier protein-dependent systems (FAS II) localized in the chloroplasts and synthesizes products with chain lengths of C16 and C18 that can be further elongated to C20–C24 (10, 11). The third, a multifunctional fatty acid synthase (FAS I) whose main products are C16 fatty acids with C14 and C18 chain lengths as minor products, is located in the cytosol of Euglena (12, 13). The fourth is a microsomal fatty acid synthase activity (14). The fifth is the mitochondrial system, which is involved in anaerobic wax ester fermentation. It uses acetyl-CoA as primer and C2-donor, a portion of the fatty acids is reduced to alcohols, esterified with another fatty acid, and deposited in the cytosol as waxes (2, 5, 15). The mitochondrial system synthesizes products of chain length 8–18 with a majority of C14 (15, 16). Synthesis of odd numbered fatty acids starts from propionyl-CoA, which is synthesized via the methylmalonyl-CoA pathway (17–19) and requires the participation of rhodinones (9).

The net biochemistry of wax ester fermentation has been characterized, but the enzymes of mitochondrial fatty acid synthesis in Euglena have not been characterized at the molecular level so far. This fatty acid synthesis has been formally described as a reversal of β-oxidation (2, 15) because it proceeds via CoA rather than acyl carrier protein-bound intermediates. But there is a key difference relative to β-oxidation: a trans-2-
trans-2-Enoyl-CoA Reductase

The following abbreviations used are: SSDH, succinate-semialdehyde dehydrogenase; GAPDH, NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase.

<table>
<thead>
<tr>
<th>Crude extract, aerobic</th>
<th>Mitochondria</th>
<th>Chloroplasts, anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>nmol mg⁻¹ min⁻¹</td>
<td>nmol mg⁻¹ min⁻¹</td>
<td>nmol mg⁻¹ min⁻¹</td>
</tr>
<tr>
<td>SSDH (n = 6)</td>
<td>18.8 ± 5.8</td>
<td>173 ± 23</td>
</tr>
<tr>
<td>GAPDH (NADP⁺) (n = 6)</td>
<td>431 ± 16</td>
<td>ND</td>
</tr>
<tr>
<td>LDH (n = 6)</td>
<td>17950 ± 1570</td>
<td>2 ± 0.8</td>
</tr>
<tr>
<td>TER NADH (n = 10)</td>
<td>5.7 ± 1.7</td>
<td>66 ± 12</td>
</tr>
<tr>
<td>TER NADPH (n = 4)</td>
<td>NM</td>
<td></td>
</tr>
</tbody>
</table>

a ND, not detectable.  
b NM, not measured.

**TABLE II**

**Purification of Euglena trans-2-enoyl-CoA reductase**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification</th>
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<tbody>
<tr>
<td></td>
<td>nmol</td>
<td>mg</td>
<td>nmol/mg/min</td>
<td>-fold</td>
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<tr>
<td>Crude extract</td>
<td>238,559</td>
<td>40,977</td>
<td>5.7</td>
<td>3</td>
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<tr>
<td>30% ammonium sulfate cut and dialysis</td>
<td>57,277</td>
<td>24,903</td>
<td>2.3</td>
<td>3</td>
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<tr>
<td>DEAE Fractogel</td>
<td>34,473</td>
<td>4536</td>
<td>7.6</td>
<td>6</td>
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<tr>
<td>Phenyl-Sepharose</td>
<td>14,666</td>
<td>1164</td>
<td>12.6</td>
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<tr>
<td>Reactive red</td>
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<td>178</td>
<td>80.1</td>
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<td>Hydroxyapatite</td>
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<td>230.6</td>
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<td>Mono Q</td>
<td>2891</td>
<td>8.4</td>
<td>323.5</td>
<td>145</td>
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<td>Native PAGE</td>
<td>587</td>
<td>0.33</td>
<td>1778.7</td>
<td>773</td>
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<tr>
<td>Superdex</td>
<td>559</td>
<td>0.144</td>
<td>3879.2</td>
<td>1687</td>
</tr>
</tbody>
</table>

**Enzyme Assay**—The assay for TER activity was performed according to Inui et al. (15) and Seubert et al. (22). The standard assay mixture contained potassium phosphate buffer, pH 6.2, 0.5 mM crotonyl-CoA (Sigma), 0.4 mM NADH, 2 μM FAD and enzyme. The assay mixture without substrate was preincubated for 10 min at 30 °C and the reaction was started with the addition of the substrate. Activity was determined by decrease of absorbance at 340 nm. The final assay volumes were 1 ml (Uitrospec 2000 Spectrophotometer, Amersham Biosciences) or 200 μl (GENios microplate reader, Tecan Instruments). Kinetic parameters of TER were estimated using substrate concentrations of 5–1000 μM for crotonyl-CoA (Sigma) and trans-2-hexenoyl-CoA, which was kindly provided by Prof. K. Hiltunen (University of Oulu, Finland). The kinetic data were transferred to Lineweaver-Burk plots to calculate kₐ values.

**Purification of Euglena trans-2-Enoyl-CoA Reductase**—All steps were performed at 4 °C with the exception of the fast protein liquid chromatography steps, which were carried out at room temperature.

*Note:* Purification step Total activity Total protein Specific activity Purification

**FIG. 1. Silver-stained 12% SDS-PAGE of Euglena TER from different steps of purification.** Lane 1, crude extract; lane 2, crude extract after 30% ammonium sulfate cut; lane 3, DEAE Fractogel eluate; lane 4, phenyl-Sepharose eluate; lane 5, reactive red eluate; lane 6, hydroxyapatite eluate; lane 7, Mono Q eluate; lane 8, native PAGE eluate; lane 9, Superdex eluate; M, molecular mass standard (sizes indicated).

Enoyl-CoA reductase (EC 1.3.1.44), designated here as TER, reduces the double bond in enoyl-CoA to produce acyl-CoA (15).

In β-oxidation this step is oxidative and irreversible under physiological conditions because acyl-CoA dehydrogenase (mitochondrial β-oxidation) and acyl-CoA oxidase (peroxisomal β-oxidation) are both linked to O₂ reduction (20, 21). Here we report the localization, purification, mass spectrometry sequencing, cloning, heterologous expression in E. coli, and kinetic parameters of TER from Euglena mitochondria.

**EXPERIMENTAL PROCEDURES**

**Medium and Culture Conditions**—Fermenter culture of E. gracilis strain Z (SAG 1224–5/25 collection of algae Göttingen) and isolation of mitochondria was performed as described (9).
Biosciences) equilibrated in buffer D. The column was washed with 15 ml of buffer D, proteins were eluted in a 20-ml gradient of 0–1 M KCl in buffer D, fractions of 280 ml were collected. Active fractions were concentrated by ultrafiltration (Amicon Ultra, Millipore), applied in six runs to a continuous, preparative 6-cm 6% native polyacrylamide gel (Mini-Prepcell, Bio-Rad), electrophoresed at 250 V, and proteins were eluted at 100 μl/min in buffer G (50 mM Tris, 25 mM borate, pH 8.7, 1 mM EDTA, 1 mM DTT, 1 μM FAD). Fractions of 200 μl were collected, fractions with TER activity were dialyzed against buffer H (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μM FAD) and loaded in three runs to a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with buffer H. Proteins were eluted with 25 ml of buffer H, fractions of 230 μl were collected and assayed for TER activity. Active fractions were visualized by SDS-PAGE and applied to in-gel digestion for subsequent ESI-Q-TOF-MS/MS analysis.

**In-gel Digestion and ESI-Q-TOF-MS/MS Analysis**—The band of the purified enzyme was cut from the gel, washed twice with 50% (v/v) acetonitrile, and incubated successively with 100% acetonitrile, 100 mM NH₄HCO₃, and 100 mM NH₄HCO₃/acetoniitrile (1:1). After vacuum drying, the gel pieces were reswollen with 10 ng μl⁻¹ trypsin (Promega) and digested for 12 h at 37 °C. Peptides were extracted in 5% (v/v) formic acid using a sonication bath. Prior to mass spectrometry, samples were desalted using C18 ZipTips (Millipore). ESI-Q-TOF-MS/MS analysis of tryptic peptides was performed with a Q-TOF mass spectrometer (Micromass).

**Hybridization Probe, Cloning, and Heterologous Expression**—Standard molecular and biochemical methods, cDNA synthesis, and cloning in λ ZAPII were performed as described (23–25). PCR was analyzed, after initial denaturation for 10 min at 95 °C, for 30 cycles with 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, final extension at 72 °C for 5 min in 25 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM magnesium acetate, 0.2 mM of each dNTP, 1 μM of each primer, 1 unit of Taq polymerase (Eppendorf), and 10–20 ng of cDNA as template. A 839-bp product was amplified with the primers 5′-GGTGGTAYAYAACIGTIGG-3′ and 5′-TATCTGGTATAGACCGTACGC-3′ (designed against the sequenced peptides 7 and 9 of purified Euglena TER). This fragment was cloned into pBluescript SK+ (Stratagene), sequenced and used as hybridization probe to screen an E. gracilis trans-2-enoyl-CoA reductase uncovered its involvement in mitochondrial fatty acid synthesis wax ester formation under anaerobic conditions (4, 15). We found roughly equal levels of NADH- and NAPDH-dependent TER activity in mitochondria isolated from both aerobically and anaerobically grown cells (Table I). The activity of the mitochondrial-specific marker enzyme of Euglena, succinate-semialdehyde dehydrogenase, was enriched 9-fold in the mitochondrial fraction versus crude extract, whereas TER was enriched even more (Table I) with no detectable TER activity in chloroplasts, suggesting that most if not all of the TER activity is localized in the mitochondrion.

In contrast to earlier purification protocols (31), we were unable to observe binding of TER to carboxymethylcellulose or other cation exchangers. We therefore established a new purification protocol starting from the Euglena crude extract. The purification procedure involves a 30% ammonium sulfate cut, anion exchange chromatography, hydrophobic interaction, affinity chromatography, hydroxyapatite chromatography, native PAGE, and gel filtration (Table II). This procedure yielded 1687-fold purification of an electrophoretically homogeneous TER preparation (Fig. 1) with a relative molecular mass of 44 kDa and a specific enzyme activity of 3879 nmol mg⁻¹ min⁻¹ (Table II). Gel filtration was applied as the final purification step.
step and the TER activity was eluted in a single peak (Fig. 2) with molecular mass of ∼44 kDa (Fig. 2), corresponding to the molecular mass in SDS-PAGE (Fig. 1), indicating that the active enzyme is a monomer. Active fractions of the last purification step were separately electrophoresed on SDS-PAGE and visualized by Coomassie staining (Fig. 3). The 44-kDa band coeluted with TER activity (Fig. 3). The 44-kDa bands (a major band and a weak doublet) were cut from the gel (marked with arrows in Fig. 3) and independently sequenced by electrospray mass spectrometry after tryptic digestion. Seventeen different internal peptide sequences were identified from purified TER protein (Table III). Below the major TER band, its weak doublet at ∼42 kDa (visible in Fig. 1) was also excised from the gel and independently sequenced; it gave the same tryptic peptides with the same amino acid sequences. The minor difference in electrophoretic mobility may be because of cofactor loss during SDS-PAGE or other electrophoretic anomaly.

Cloning of Euglena TER—The 17 peptides showed no significant similarity to sequenced proteins in database searches or to 2700 expressed sequence tags contigs generated in-house from Euglena, leaving the orientation of the tryptic peptides in the protein unknown. Degenerate primers corresponding to peptides 5, 6, 7, 9, 11, and 12, each with forward and reverse direction were used for PCR against Euglena cDNA. Peptide 7 (forward) and peptide 9 (reverse) primers amplified a 839-bp product, the sequence of which contained 10 additional peptides determined from purified TER (Fig. 4), identifying the probe as TER-specific.

FIG. 3. Coomassie-stained 12% SDS-PAGE of separate, active fractions after final chromatography on Superdex 200 HR 10/30 column. Each lane contains 65 μl of the 280-μl fractions. Arrows indicate the bands excised for analysis by electrospray mass spectrometry. Determined TER activities of each fraction are shown above the corresponding lane.
Screening of 250,000 recombinant phages in a *Euglena* cDNA library yielded five independent clones of differing length from each transcript (Fig. 4). One clone, pEgTER1, contained a full-length cDNA as indicated by the presence of a spliced leader at the 5' end (32), was 1912 bp long with an open reading frame (ORF) of 1617 nucleotides encoding a protein of 559 amino acid residues (Fig. 4). Northern hybridization revealed that *Euglena* TER was expressed under both aerobic and anaerobic conditions (Fig. 5).

**TER cDNA Expressed in E. coli Is Active—**Data base comparisons (see next section) revealed that the C-terminal 405 aa encoded by pEgTER1 were strongly conserved with proteins of unknown function from sequenced prokaryotic genomes. The complete ORF of pEgTER1 in addition to the 405-aa C-terminal region were amplified and cloned into expression vectors pET28a and pET32a and transformed into *E. coli* BL21(DE3) and *E. coli* Rosetta(DE3). Induction by the addition of 0.4 mM isopropyl-1-thio-

### Table III

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Sequenced peptide</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>ACLKPLGATTTNR</td>
</tr>
<tr>
<td>2</td>
<td>AALEAGLYAR</td>
</tr>
<tr>
<td>3</td>
<td>VLVLCGSTQGYLSTR</td>
</tr>
<tr>
<td>4</td>
<td>TDPAT</td>
</tr>
<tr>
<td>5</td>
<td>SLDGDAFSTDKT</td>
</tr>
<tr>
<td>6</td>
<td>DLWSQVANTALK</td>
</tr>
<tr>
<td>7</td>
<td>AGWYNTVAFK</td>
</tr>
<tr>
<td>8</td>
<td>RVQUEELAYAR</td>
</tr>
<tr>
<td>9</td>
<td>DLSDFACQYTEFLR</td>
</tr>
<tr>
<td>10</td>
<td>LYPQGDSPVLDEAGR</td>
</tr>
<tr>
<td>11</td>
<td>LTQYYGCPAYPVVAK</td>
</tr>
<tr>
<td>12</td>
<td>VDWWMAEDVQQAVK</td>
</tr>
<tr>
<td>13</td>
<td>STGYGMV/LSEK</td>
</tr>
<tr>
<td>14</td>
<td>AHQPTSGPK</td>
</tr>
<tr>
<td>15</td>
<td>ALSEAEGLAQQK</td>
</tr>
<tr>
<td>16</td>
<td>((GT/AAS)/)HEGQLEMQMR</td>
</tr>
<tr>
<td>17</td>
<td>LYPENGAPLVDSEQR</td>
</tr>
</tbody>
</table>

of 1452 ± 24 nmol mg⁻¹ min⁻¹ with hexonol-CoA. The recombinant enzyme was also active with NADPH as co-factor, showing specific activities of 658 ± 9.2 and 374 ± 10.4 nmol mg⁻¹ min⁻¹ for crotonyl-CoA and hexenyl-CoA, respectively.

**TER of Euglena Defines a New Gene Family in Genomes—**Data base searching with the 134 N-terminal amino acids of Euglena TER returned no significant hits, but searching with the 405 C-terminal residues of TER returned 74 prokaryotic sequences with a match better than 10⁻³⁰, none of which had an annotated function. The phylogenetic distribution of the hits was notable: 33 from γ-proteobacteria, five from β-proteobacteria, two from actinobacteria, two from *Clostridia*, one from Spirochaetes, one from Cytophaga, and 30 from environmental sequences. A phylogenetic network showing sequence similarities among a representative sample of TER homologues relative to the sequence of the active *Euglena* enzyme is shown in Fig. 8.

The only data base hit to a protein in a gene cluster of annotated function was a match to the sequence AB070934 of the *Streptomyces avermitilis* polyketide biosynthetic cluster 5, *pks5* (33). The *S. avermitilis* TER homologue (E-value 10⁻⁵⁰) is located as a 439-aa hypothetical protein with 40% amino acid identity to *Euglena* TER directly downstream of a modular (Type I) polyketide synthase (33). Although there are eight type I polyketide biosynthesis clusters in the *S. avermitilis* genome (33), the TER homologue occurs only once in that genome. Comparison of the genomic region surrounding the TER homologues from sequenced genomes using the CMR resource at TIGR (tigr.org/tdb/) revealed that TER homologues are occasionally situated downstream and on the same strand of proteins with annotations suggesting a role in fatty acid metabolism, such as acyl-CoA dehydrogenase or 3-oxoacyl-(acyl carrier protein) reductase (Fig. 9). No functional annotation has been previously assigned to any TER homologue, but a role for these proteins in reducing the double bond in enoyl-CoA, like TER, or possibly enoyl-acyl carrier protein, seems possible.

**DISCUSSION**

The physiological significance of the *Euglena* mitochondrial fatty acid biosynthesis system is that it operates directly with acetyl-CoA rather than with malonyl-CoA, thereby circumventing the ATP-consuming activation step catalyzed by acetyl-CoA carboxylase (2, 15). This saving of one ATP per pyruvate in- vested in fatty acid biosynthesis permits a net gain of 2 ATP per glucose from glycolysis with acetyl-CoA reduction, consuming NADH from the glyceraldehyde-3-phosphate dehydrogenase step of glycolysis and NADPH from the pyruvate:NADP⁺ oxidoreductase and/or pyruvate dehydrogenase complex steps of mitochondrial pyruvate oxidation, to yield esterified fatty acids (wax ester fermentation). TER catalyzes a critical reducing step, the reverse reaction of the O₂-dependent step of β-oxidation, in acyl-CoA-dependent mitochondrial fatty acid synthesis of *Euglena*.

The TER activity found in isolated mitochondria of *Euglena* (Table I) was purified to homogeneity from the crude extract of 1 kg of *Euglena* cells. Active TER is a monomer with a molecular mass of 44 kDa. This finding is in marked contrast to the previous report of a heteromeric composition of *Euglena* TER consisting of 15- and 25-kDa subunits (31), the further characterization of which has not been reported. Purified *Euglena* TER showed a specific activity of 3880 nmol mg⁻¹ min⁻¹, comparable with the value of 1944 nmol mg⁻¹ min⁻¹ previously reported (31). The sequences of the 17 tryptic peptides determined by ESI-Q-TOF MS/MS from electrophoretically homogenous TER purified from *Euglena* allowed us to clone the corresponding cDNA, the C-terminal 405 aa of which were expressed in *E. coli* and possessed TER activity comparable...
with that of the purified enzyme. These findings attribute trans-2-enoyl-CoA reductase activity (EC 1.3.1.44) to the Euglena TER sequence, the first enzymatically characterized member of a novel protein family widespread among genomes.

The Km values for recombinant TER show that the enzyme can catalyze reduction of trans-2-enoyl-CoA substrates with chain lengths of C4 and C6. The km for trans-hexenoyl-CoA was 1.3

**FIG. 4.** Full-length cDNA sequence for TER from E. gracilis. Non-coding regions are indicated in lower cases. Spliced leader sequence is printed in italics. Arrowheads indicate starts of further identified independent TER cDNA clones. PCR amplicon used as the screening probe is underlined in the nucleotide sequence. Peptide sequences from mass spectrometry sequencing of purified enzyme are underlined in the deduced amino acid sequence. Start of the second construct used for heterologous expression in E. coli is printed in bold. Potential binding and catalytic domains are marked in gray. Residues 190–196, NAD(P)H-binding domain; residues 231–238 and 279–285, catalytic domains; residues 515–520, FAD-binding domain.

**FIG. 5.** Northern blot of E. gracilis poly(A)+ mRNA (5 µg/lane) probed with full-length TER cDNA. Lane 1, mRNA isolated from aerobically grown cells; lane 2, mRNA isolated from anaerobically grown cells. The band is 1.9 kb; no additional bands were detected.

**FIG. 6.** Western blot analysis with immunodetection of the complete TER open reading frame in pET32a expressed in Rosetta (DE3). Detection was carried out with anti-His antibody (monoclonal mouse IgG, Novagen). Anti-mouse secondary antibody horseradish peroxidase conjugate from goat was used and the signal was visualized with ECL Western blotting analysis system (Amersham Biosciences). Lane 1, soluble bacterial fraction. Lanes 2–9 show different fractions after Ni-NTA purification: lane 2, flow-through; lane 3, first wash; lane 4, second wash; lane 5, third wash; lane 6, first elution; lane 7, second elution; lane 8, third elution; lane 9, fourth elution.

**FIG. 7.** Western blot analysis with immunodetection of the 405-aa C-terminal part of the TER cDNA clone in pET28a expressed in BL21(DE3). Detection was carried out with anti-His antibody (monoclonal mouse IgG, Novagen). Anti-mouse secondary antibody horseradish peroxidase conjugate from goat was used and the signal was visualized with the ECL Western blotting Analysis System (Amersham Biosciences). Lane 1, soluble bacterial fraction. Lanes 2–8 show different fractions after Ni-NTA purification: lane 2, flow-through; lane 3, first wash; lane 4, second wash; lane 5, first elution; lane 6, second elution; lane 7, third elution; lane 8, fourth elution.
times higher than that for crotonyl-CoA. Both substrates were reduced with either NADH or NADPH as cofactor but the specific activities with NADPH as cofactor were up to 3.8 times lower than with NADH.

Edman sequencing of the enzyme purified from *Euglena* revealed that the N terminus was blocked (data not shown), so that the exact processing site of the mitochondrial transit peptide could not be directly determined. However, the predicted molecular mass of the 405-aa active product expressed in *E. coli* was 43,784 Da, as compared with the molecular mass of ~45 kDa observed in SDS-PAGE for the expressed protein (Fig. 7) and ~44 kDa for the active TER enzyme purified from *Euglena* (Fig. 1). This indicates that the processing site of the transit peptide and the N terminus of the mitochondrial protein is close to motif MAMFTT indicated in Fig. 4. The plasmid pEgTER1 contains an ORF of 539 aa with a calculated molecular mass of 59 kDa, substantially larger than the molecular mass of purified TER (44 kDa). *Euglena* mitochondrial targeting sequences do not belong to the learning set employed by targeting prediction programs such as MitoprotII (34) and iP-SORT (35) and only a few transit peptides of mitochondrial proteins are known, which are about 30–40-aa long (8, 36). Thus, the exact role of the 135 N-terminal aa in the ORF of pEgTER1 is unclear as is the identity of the start codon used in *E. coli* showed no need for exogenous FAD in the enzyme assay. Variation of FAD concentration in the assay (0–5 μM) FAD had no effect on activity of the recombinant enzyme either with crotonyl-CoA or trans-hexenoyl-CoA as substrate (data not shown). FAD might therefore stabilize the TER enzyme purified here, but does not seem essential for catalysis. Additionally, two putative catalytic sites of the short-chain dehydrogenase/reductase family were identified in the TER amino acid sequence. Most proteins of this family possess a catalytic site with consensus motif MMXK (37, 40, 41), and some show a modified motif with YYXXK (42, 43). TER from *Euglena* possesses the motif YYXK at residues 231–238 and YYXK at residues 279–285 (Fig. 4). In general, enoyl thioester reductases constitute a heterologues family with regard to their amino acid sequence but the majority of known enoyl thioester reductases belong to the short-chain dehydrogenases/reductases superfamily of proteins (44). However, two enoyl thioester reductases recently characterized from *Candida tropicalis* (Etr1p) and *Saccharomyces cerevisiae* (Mf1p) were identified that belong to the family of medium chain dehydrogenases/reductases (45, 46).
Similar Biochemical Steps Encoded by Different Enzymes—An adaptation to anaerobic conditions similar to Euglena mitochondria is known from the parasitic nematode Ascaris suum. Early larval stages of Ascaris are aerobic and mitochondria possess a functional TCA cycle and cytochrome c oxidase (47, 48), whereas adults use organic acids instead of oxygen as terminal electron acceptors (49, 50). Main end products of anaerobic energy metabolism of Ascaris mitochondria are the methyl-branched fatty acids 2-methylbutyrate and 2-methylvalerate (51). These reactions of anaerobic mitochondrial metabolism resemble Euglena mitochondrial fatty acid synthesis in that acetyl-CoA and propionyl-CoA condensations are involved and the final reduction is catalyzed by a 2-methyl branched chain enoyl-CoA reductase (51, 52). However, the Ascaris 2-methyl branched chain enoyl-CoA reductase does not accept NADH as electron donor, in contrast to Euglena TER, but does accept electrons from an electron transporting flavoprotein and shows sequence similarities to acyl-CoA dehydrogenases (53–55). Euglena TER is clearly distinct from the Ascaris enzyme.

An NADPH-specific crotonyl-CoA reductase from Streptomyces cohnii catalyzes the synthesis of butyryl-CoA from crotonyl-CoA (56), a reaction very similar to that catalyzed by Euglena TER. However, no detectable sequence similarity exists between TER and crotonyl-CoA reductase. Furthermore, S. cohnii crotonyl-CoA reductase shows no activity with NADH in contrast to Euglena TER (56, 57). Crotonyl-CoA reductase provides butyryl-CoA units for synthesis of polyketids (58), but is again distinct from Euglena TER.

A variety of enoyl reductases and enoyl reductase modules are known among polyketide synthases (59, 60), but none characterized so far have sequence similarity with Euglena TER. Northern blot analysis of TER confirmed the determination of TER activity of isolated mitochondria (Fig. 5, Table I). TER is expressed both under aerobic and anaerobic conditions, in line with findings from other anaerobic mitochondria, which seem to have adopted a strategy of being prepared for anaerobiosis without the need for specific enzyme induction (9, 61).

A Function in a Family of Otherwise Hypothetical Proteins—Euglena TER has strong sequence similarity to proteins annotated as hypothetical reading frames in several dozen prokaryotes, most conspicuously among H9253-proteobacteria. The operon context of TER homologues from sequenced genomes revealed that TER homologues (annotated so far as hypothetical reading frames) most commonly occur next to other hypothetical reading frames, but sometimes occur downstream of enzymes associated with fatty acid biosynthetic pathways and in one case downstream of a polyketide synthase in the S. avermitilis genome (Fig. 9). This raises the possibility that TER homologues (and Euglena TER itself) could operate in fatty acid biosynthesis, polyketide biosynthesis, or conceivably both, because both pathways involve thioester-bound enoyl reduction. The finding that TER is an active monomer suggests that catalysis of the NADH- and NADPH-dependent reduction of the double bond in crotonyl-CoA does not require interactions with other proteins.

What Is the Biosynthetic Route to Wax Esters in Euglena?—Fatty acid biosynthesis catalyzed by FAS complexes and polyketide biosynthesis catalyzed by polyketide synthases involve four fundamentally similar steps: (i) the condensation of an acetyl moiety onto a acyl thioester to form a β-ketoacyl thioester, (ii) reduction of the keto group to a β-hydroxyacyl group by a reduction due to a 2-methyl branched chain enoyl-CoA reductase, (iii) further elongations and thioesterifications, and (iv) the formation of a fatty acid thioester in the final step.

FIG. 9. Operon analysis of homologues of Euglena TER via the CMR resource at TIGR. Open arrows show ORFs involved in fatty acid biosynthetic pathways, and hatched arrows indicate miscellaneous functions.
thioester, (iii) dehydrogenation to form the double bond in the resulting enoyl thioester, and (iv) reduction of the double bond to form an acyl thioester two carbons longer than the first one. The thiol moiety of the thioester is pantothenate, which is covalently bound to acyl carrier protein in polyketide synthases and FAS complexes (62, 63). Fatty acid elongases, which extend covalently bound to acyl carrier protein in polyketide synthases result in the common eukaryotic ancestor (71–73), whereby the view that donor was the ancestral mitochondrion or whether it comes from the mitochondrion, then additional eukaryotic lineages should eventually be found that harbor the same gene.

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