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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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Meike Hoffmeister‡, Markus Piotrowski§, Ulrich Nowitzki‡, and William Martin‡¶

From the ‡Institute of Botany III, University of Düsseldorf, D-40225 Düsseldorf, Germany and the §Department of Plant Physiology, University of Bochum, D-44801 Bochum, Germany

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 1.3.1.44), 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 succinate-semialdehyde shunt as found in the α-proteobacerium *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:$NADP^+$ 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)[1] localized in the chloroplasts and synthesize 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 rhodoquinones (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-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY741582.

¶ To whom correspondence should be addressed: Institute of Botany III, University of Düsseldorf, D-40225 Düsseldorf, Germany. Tel.: 49-211-811-3011; Fax: 49-211-811-3554; E-mail: w.martin@uni-duesseldorf.de.

[1] The abbreviations used are: FAS, fatty acid synthase; TER, *trans*-2-enoyl-CoA reductase; ESI-Q-TOF-MS/MS, electrospray ionization quadruple time-of-flight tandem mass spectrometry; ORF, open reading frame; Ni-NTA, nickel nitrilotriacetic acid; DTT, dithiothreitol; aa, amino acid(s).

TABLE I
Measurement of marker enzymes and TER in cell fractions from E. gracilis ± S.D.

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

	Crude extract, aerobic	Mitochondria		Chloroplasts, anaerobic
		Aerobic	Anaerobic	
	nmol mg^{-1} min^{-1}	*nmol mg^{-1} min^{-1}*	*nmol mg^{-1} min^{-1}*	*nmol mg^{-1} min^{-1}*
SSDH (n = 6)	18.8 ± 5.8	173 ± 23	154 ± 13	ND[a]
GAPDH ($NADP^+$) (n = 6)	431 ± 16	ND	ND	5046 ± 320
LDH (n = 6)	17950 ± 1570	2 ± 0.8	6 ± 0.7	ND
TER NADH (n = 10)	5.7 ± 1.7	63.5 ± 12	66.3 ± 12	ND
TER NADPH (n = 4)	NM[b]	86.4 ± 13	77.6 ± 28	NM

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

TABLE II
Purification of Euglena trans-2-enoyl-CoA reductase

Purification step	Total activity	Total protein	Specific activity	Purification
	nmol	*mg*	*nmol/mg/min*	*-fold*
Crude extract	233,569	40,977	5.7	
30% ammonium sulfate cut and dialysis	57,277	24,903	2.3	
DEAE Fractogel	34,473	4536	7.6	3
Phenyl-Sepharose	14,666	1164	12.6	6
Reactive red	14,258	178	80.1	35
Hydroxyapatite	13,375	58	230.6	100
Mono Q	2801	8.4	333.5	145
Native PAGE	587	0.33	1778.7	773
Superdex	559	0.144	3879.2	1687

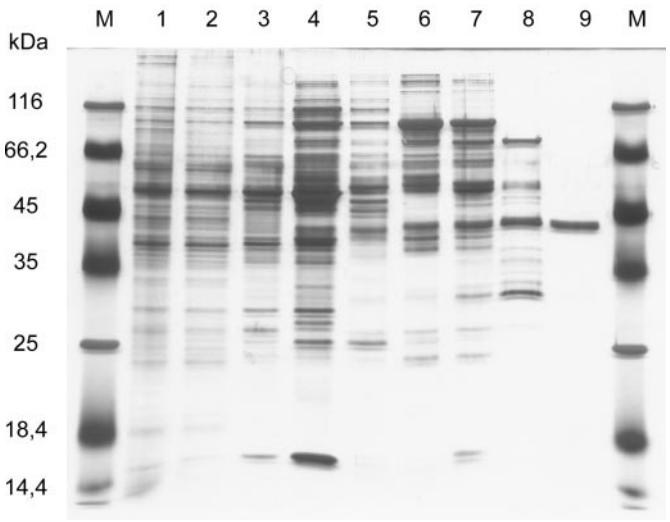

FIG. 1. **Silver-stained 12% SDS-PAGE of *Euglena* TER from different steps of purification.** *Lane 1*, crude extract; *lane 2*, crude extract after 30% ammonium sulfate cut; *lane 3*, DEAE Fractogel eluate; *lane 4*, phenyl-Sepharose eluate; *lane 5*, reactive red eluate; *lane 6*, hydroxyapatite eluate; *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 O_2 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).

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 (Ultrospec 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 k_m 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. One kg of *Euglena* cells were homogenized in buffer A (25 mM HEPES-NaOH, pH 7.4, 0.25 M sorbitol, 1 mM EDTA, 1 mM DTT, 1 μM FAD) using a French Press and centrifuged at 1500 × *g* for 15 min. The supernatant of a 30% ammonium sulfate cut was dialyzed against buffer B (25 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 1 mM DTT, 1 μM FAD) to <6 μS/cm and applied in 10 runs to a 2.6 × 12-cm DEAE Fractogel EMD 650 S column (Merck). The column was washed with 180 ml of buffer B, proteins were eluted in a 100-ml gradient of 0–1 M KCl in buffer B, fractions of 2 ml were collected. Fractions with TER activity were pooled, dialyzed against buffer C (10 mM Tris-HCl pH 8.0, 1 M $NH_4(SO_4)_2$, 1 mM EDTA, 1 mM DTT, 1 μM FAD), and loaded in four runs to a 2.6 × 14-cm Phenyl-Sepharose 6 FF column (Amersham Biosciences) equilibrated with buffer C. The column was washed with 140 ml of buffer C, proteins were eluted in a 140-ml gradient of 1–0 M $NH_4(SO_4)_2$ in buffer D (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 1 μM FAD). Fractions (2.5 ml each) with TER activity were pooled, dialyzed against buffer B, and bound in five runs to a 1.6 × 9-cm Reactive Red 120 column (Sigma) equilibrated with buffer B. The column was washed with 100 ml of buffer B, proteins were eluted in a 200-ml gradient of 0–1 M KCl in buffer B, fractions of 3 ml were collected. Active fractions were pooled and dialyzed against buffer E (10 mM potassium phosphate buffer, pH 6.8, 1 mM DTT, 1 μM FAD) and applied in eight runs to a Eco-PacCHT II Cartridge (Bio-Rad) equilibrated with buffer E. The column was washed with 15 ml of buffer E, proteins were eluted in a 25-ml gradient of buffer E to buffer F (500 mM potassium phosphate buffer, pH 6.8, 1 mM DTT, 1 μM FAD). Fractions with TER activity (1 ml each) were pooled, dialyzed against buffer D, and bound in three runs to a Mono Q HR 5/5 column (Amersham

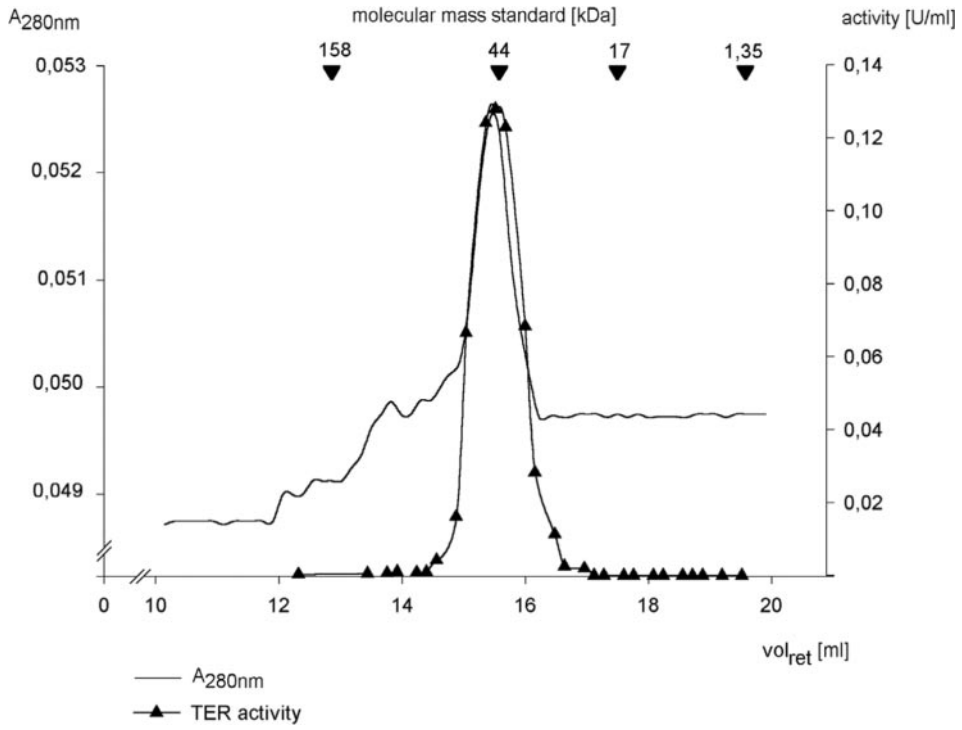

FIG. 2. **Elution profile of TER after chromatography on a Superdex 200 HR 10/30 column.** Elution was carried out with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 μM FAD. Fraction size was 230 μl.

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 μl 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 NH_4HCO_3, and 100 mM NH_4HCO_3/acetonitrile (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-TOF2 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 performed, after initial denaturation for 10 min at 98 °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′-GGITGGTAYA-AYACIGTIGC-3′ and 5′-GTYTCRTAICCIGCRAARTC-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 *Euglena* cDNA library. Five independent positives were isolated and sequenced, all five represented the same transcript and varied only in length. The insert of one plasmid so derived, pEgTER1, was sequenced completely double-stranded via deletion by exonuclease III (26). Two different parts of the pEgTER1 were chosen for heterologous expression in *E. coli*. The two constructs were amplified from *Euglena* cDNA as described above using the primers: TER1Ndefor, 5′-TATACATATGTCGTGCCCCGCCTCGCCGTCTG-3′; TER1Bglfor, 5′-TATAGATCTTATGTCGTGCCCCGCCTCGCCGTC-TG-3′; TER2Ndefor, 5′-TATACATATGTTCACCACCACAGCGAAGGT-CATCC-3′; and TERXhorev, 5′-TATCTCGAGCTACTGCTGGGCAGCA-CTGG-3′ to introduce 5′-NdeI, respectively, 5′-BglII and 3′-XhoI restrictions sites. The amplification products were cloned into pET28a or pET32a (Novagen), cut with the same restriction enzymes, and expressed in the *E. coli* expression strain BL21(DE3) or Rosetta(DE3) (both Novagen).

Phylogenetic Network—Homologues were retrieved and aligned using ClustalW (27). Protein LogDet distances were calculated with the program LDDist (28). NeighborNet planar graphs of LogDet distances were constructed with NNet (29) and visualized with Splitstree (30).

RESULTS

Purification and Microsequencing of Euglena TER—Early work on *E. gracilis trans*-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

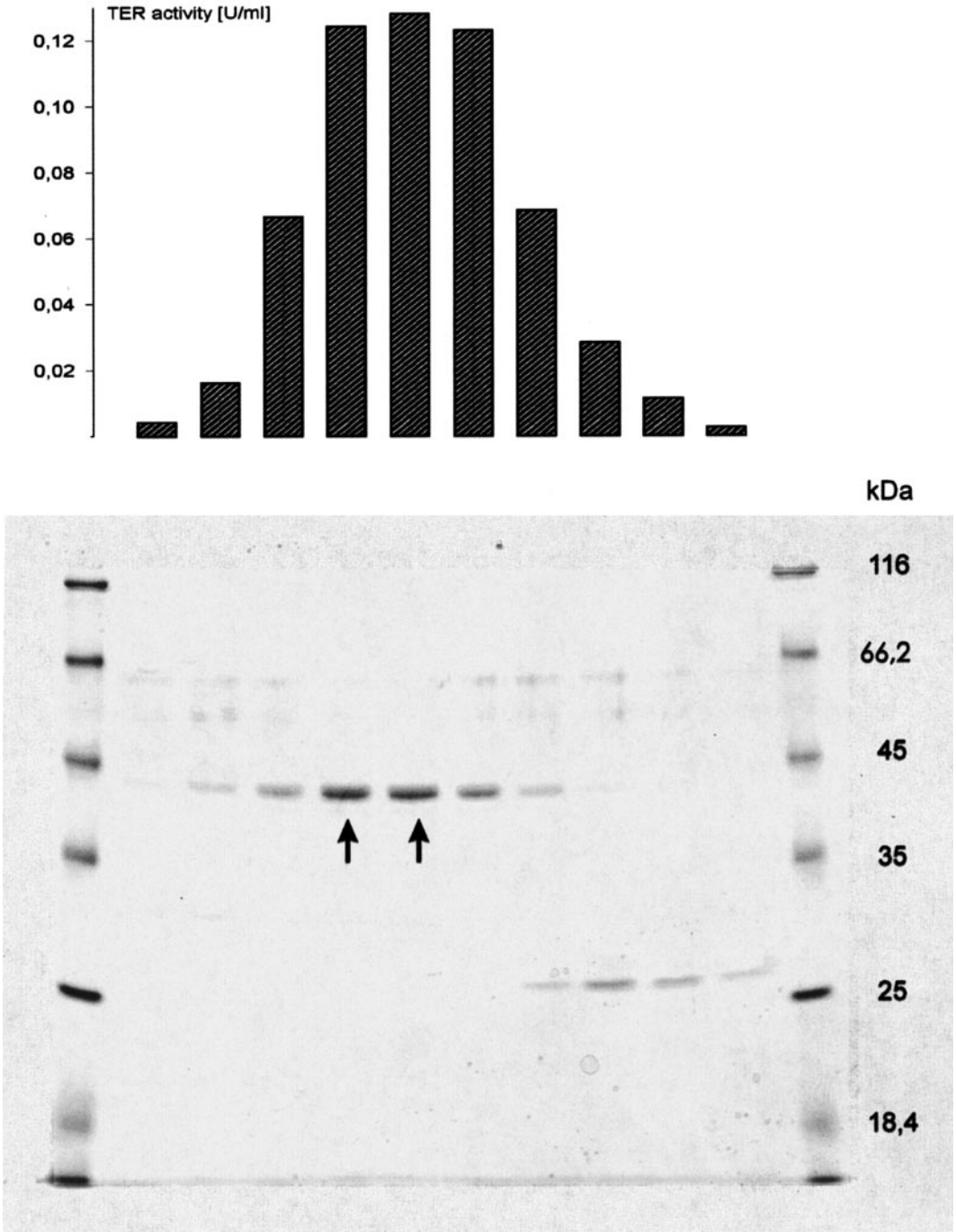

FIG. 3. **Coomassie-stained 12% SDS-PAGE of separate, active fractions after final chromatography on Superdex 200 HR 10/30 column.** Each lane contains 65 μl of the 280-μl fractions. *Arrows* indicate the bands excised for analysis by electrospray mass spectrometry. Determined TER activities of each fraction are shown *above* the corresponding lane.

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 data base 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.

TABLE III
Internal peptide sequences of purified trans-2-enoyl-CoA reductase identified by electrospray mass spectrometry sequencing of tryptic peptides

Peptide no.	Sequenced peptide
1	ACLKPLGATYTNR
2	AALEAGLYAR
3	VLVLGCSTGYGLSTR
4	TDPAT
5	SLDGDAFDSTTK
6	DLWSQVNTANLK
7	AGWYNTVAFEK
8	RVQEELAYAR
9	DLSDFAGYQTEFLR
10	LYPGDGSPLVDEAGR
11	LTQQYGCPAYPVVAK
12	VDDWEMAEDVQQAVK
13	STGYG(AMVR/LSEK)
14	AHPPTSPGPK
15	ALSEAGVLAEQK
16	((GT)/(AS))HEGCLEQMVR
17	LYPENGAPLVDEQR

Screening of 250,000 recombinant phages in a *Euglena* cDNA library yielded five independent clones of differing length from the same transcript (Fig. 4). One clone, pEgTER1, contained a full-length cDNA as indicated by the presence of a 5′-TTTTCG-3′ spliced leader at the 5′ end (32), was 1912 bp long with an open reading frame (ORF) of 1617 nucleotides encoding a protein of 539 amino acid residues (Fig. 4). Northern hydridization 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-β-D-galactopyranoside at 16 °C overnight yielded expression of the complete TER ORF in pET32a and Rosetta(DE3) as shown by immunodetection of the His tag in the soluble fraction and after purification by Ni-NTA chromatography (Fig. 6). The detected band shows the expected size of 78 kDa, corresponding to the open reading frame of 59 kDa plus 19 kDa of the thioredoxin tag, but the protein was enzymatically inactive. However, when the C-terminal region of pEgTER1 beginning with Met^{135} (Fig. 4) was expressed in pET28a with BL21(DE3) as revealed by immunodetection of the His tag, the resulting protein was both soluble and active, also after Ni-NTA purification (Fig. 7). The reasons why the complete ORF was inactive while the Ni-NTA-purified 405-aa construct did possess high TER-specific activity are not fully clear. But because the 405-aa construct lacks the transit peptide-coding region needed to target the protein into mitochondria and has nearly the same molecular mass (45 kDa, Fig. 7) as the protein purified from *Euglena* (44 kDa, Fig. 1), it appears that the transit peptide-coding region present in the complete ORF interferes with enzymatic activity (see "Discussion").

Catalytic Properties of Recombinant TER—The kinetic parameters of TER were determined using the 405-aa construct expressed in *E. coli*. The k_m values were 68 ± 3.9 μM for crotonyl-CoA (*trans*-2-butenoyl-CoA) and 91 ± 1.8 μM for *trans*-2-hexenoyl-CoA using NADH as the cofactor. A fixed concentration of 500 μM crotonyl-CoA was used to determine the k_m values of 109 ± 3.7 μM for NADH and of 119 ± 1.3 μM for NADPH. The recombinant enzyme possessed a NADH-specific activity of 1648 ± 17 nmol mg^{-1} min^{-1} with crotonyl-CoA as substrate and of 1452 ± 24 nmol mg^{-1} min^{-1} with hexenoyl-CoA. The recombinant enzyme was also active with NADPH as cofactor, showing specific activities of 658 ± 9.2 and 374 ± 10.4 nmol mg^{-1} min^{-1} for crotonyl-CoA and hexenoyl-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^{-20}, 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^{-69}) 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 invested 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 NAD(P)H 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 reductive step, the reverse reaction of the O_2-dependent step of β-oxidation, in acetyl-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 homogeneous 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

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  >>          >           >
ttttcgcccgtgcaccacgATGTCGTGCCCCGCCTCGCCGTCTGCTGCCGTGGTGTCTGC   60
                  M  S  C  P  A  S  P  S  A  A  V  V  S  A     14

CGGCGCCCTCTGCCTGTGCGTGGCAACGGTATTGTTGGCGACTGGATCCAACCCCACCGC  120
 G  A  L  C  L  C  V  A  T  V  L  L  A  T  G  S  N  P  T  A    34

CCTGTCCACTGCTTCCACTCGCTCTCCGACCTCACTGGTCCGTGGGGTGGACAGGGGCTT  180
 L  S  T  A  S  T  R  S  P  T  S  L  V  R  G  V  D  R  G  L    54

GATGAGGCCAACCACTGCAGCGGCTCTGACGACAATGAGAGAGGTGCCCCAGATGGCTGA  240
 M  R  P  T  T  A  A  A  L  T  T  M  R  E  V  P  Q  M  A  E    74

GGGATTTTCAGGCGAAGCCACGTCTGCATGGGCCGCCGCGGGGCCGCAGTGGGCGGCGCC  300
 G  F  S  G  E  A  T  S  A  W  A  A  A  G  P  Q  W  A  A  P    94

GCTCGTGGCCGCGGCCTCCTCCGCACTGGCGCTGTGGTGGTGGGCCGCCCGGCGCAGCGT  360
 L  V  A  A  A  S  S  A  L  A  L  W  W  W  A  A  R  R  S  V   114

GCGGCGGCCGCTGGCAGCGCTGGCGGAGCTGCCCACCGCGGTCACCCACCTGGCCCCCCC  420
 R  R  P  L  A  A  L  A  E  L  P  T  A  V  T  H  L  A  P  P   134

GATGGCGATGTTCACCACCACAGCGAAGGTCATCCAGCCCAAGATTCGTGGCTTCATCTG  480
 M  A  M  F  T  T  T  A  K  V  I  Q  P  K  I  R  G  F  I  C   154

CACGACCACCCACCCGATCGGCTGTGAGAAGCGGGTCCAGGAGGAGATCGCGTACGCCCG  540
 T  T  T  H  P  I  G  C  E  K  R  V  Q  E  E  I  A  Y  A  R   174
                                  peptide 8
TGCCCACCCGCCCACCAGCCCTGGCCCGAAGAGGGTGCTGGTCATCGGCTGCAGTACCGG  600
 A  H  P  P  T  S  P  G  P  K  R  V  L  V  I  G  C  S  T  G   194
       peptide 14                          peptide 3
CTACGGGCTCTCCACCCGCATCACCGCTGCCTTCGGCTACCAGGCCGCCACGCTGGGCGT  660
 Y  G  L  S  T  R  I  T  A  A  F  G  Y  Q  A  A  T  L  G  V   214

GTTCCTGGCGGGCCCCCCGACGAAGGGCCGCCCCGCCGCGGCGGGCTGGTACAACACCGT  720
 F  L  A  G  P  P  T  K  G  R  P  A  A  A  G  W  Y  N  T  V   234
                                                peptide 7
GGCGTTCGAGAAGGCCGCCCTGGAGGCCGGGCTGTACGCCCGGAGCCTTAATGGCGACGC  780
 A  F  E  K  A  A  L  E  A  G  L  Y  A  R  S  L  N  G  D  A   254
                   peptide 2                    peptide 5
CTTCGACTCCACAACGAAGGCGCGGACGGTCGAGGCGATCAAGCGGGACCTCGGCACGGT  840
 F  D  S  T  T  K  A  R  T  V  E  A  I  K  R  D  L  G  T  V   274

GGACCTCGTGGTGTACAGCATCGCCGCCCCGAAGCGGACGGACCCTGCCACCGGCGTCCT  900
 D  L  V  V  Y  S  I  A  A  P  K  R  T  D  P  A  T  G  V  L   294
                                  peptide 4
CCACAAGGCCTGCCTGAAGCCCATCGGCGCCACGTACACCAACCGCACTGTGAACACCGA  960
 H  K  A  C  L  K  P  I  G  A  T  Y  T  N  R  T  V  N  T  D   314
                peptide 1
CAAGGCGGAGGTGACCGACGTCAGCATTGAGCCGGCCTCCCCCGAAGAGATCGCGGACAC 1020
 K  A  E  V  T  D  V  S  I  E  P  A  S  P  E  E  I  A  D  T   334

GGTGAAGGTGATGGGCGGGGAGGACTGGGAGCTCTGGATCCAGGCGCTGTCGGAGGCCGG 1080
 V  K  V  M  G  G  E  D  W  E  L  W  I  Q  A  L  S  E  A  G   354
                                  peptide 15
CGTGCTGGCGGAGGGGGCCAAGACGGTGGCGTACTCCTACATCGGCCCCGAGATGACGTG 1140
 V  L  A  E  G  A  K  T  V  A  Y  S  Y  I  G  P  E  M  T  W   374

GCCTGTCTACTGGTCCGGCACCATCGGGGAGGCCAAGAAGGACGTGGAGAAGGCTGCCAA 1200
 P  V  Y  W  S  G  T  I  G  E  A  K  K  D  V  E  K  A  A  K   394

GCGCATCACGCAGCAGTACGGCTGCCCGGCGTACCCGGTGGTGGCCAAGGCCTTGGTCAC 1260
 R  I  T  Q  Q  Y  G  C  P  A  Y  P  V  V  A  K  A  L  V  T   414
                peptide 11
CCAGGCCAGCTCCGCCATCCCGGTGGTGCCGCTCTACATCTGCCTGCTGTACCGCGTTAT 1320
 Q  A  S  S  A  I  P  V  V  P  L  Y  I  C  L  L  Y  R  V  M   434

GAAGGAGAAGGGCACCCACGAGGGCTGCATCGAGCAGATGGTGCGGCTGCTCACCACGAA 1380
 K  E  K  G  T  H  E  G  C  I  E  Q  M  V  R  L  L  T  T  K   454
                   peptide 16
GCTGTACCCCGAGAACGGGGCCCCCATCGTCGATGAGGCCGGACGTGTGCGGGTGGATGA 1440
 L  Y  P  E  N  G  A  P  I  V  D  E  A  G  R  V  R  V  D  D   474
                peptide 10
CTGGGAGATGGCGGAGGATGTGCAGCAGGCTGTTAAGGACCTCTGGAGCCAGGTGAGCAC 1500
 W  E  M  A  E  D  V  Q  Q  A  V  K  D  L  W  S  Q  V  S  T   494
          peptide 12                          peptide 6
TGCCAACCTCAAGGACATCTCCGACTTCGCTGGGTATCAAACTGAGTTCCTGCGGCTGTT 1560
 A  N  L  K  D  I  S  D  F  A  G  Y  Q  T  E  F  L  R  L  F   514
                      peptide 9
CGGGTTCGGCATTGACGGCGTGGACTACGACCAGCCCGTGGACGTGGAGGCGGACCTCCC 1620
 G  F  G  I  D  G  V  D  Y  D  Q  P  V  D  V  E  A  D  L  P   534

CAGTGCTGCCCAGCAGTAGgtgctggacgccgcctctctccgggggtctgccaaaatgg  1680
 S  A  A  Q  Q  *                                             539

tcgctcccccaacccaaccccctgcccaccatcggggtcccgcgggtgaatgcggccccc  1740

acccaaaggcaaaggtcaaggccggggccccaccgccaaagggtaacacatatgtatccg  1800

tcgggggctgatccgcgtgcgacacgggccataattgtgccccacgggatgtccatgcgc  1860

ctaagacaactgccccggccgagagtcgctaccgccttgagttccccaggca         1912
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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.

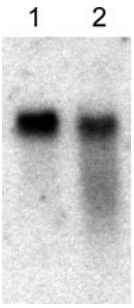

FIG. 5. **Northern blot of *E. gracilis* poly(A)$^+$ mRNA (5 μg/lane) probed with full-length TER cDNA.** *Lane 1*, mRNA isolated from aerobically grown cells; *lane 2*, mRNA isolated from anaerobically grown cells. The band is 1.9 kb; no additional bands were detected.

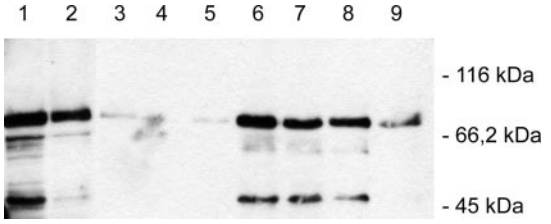

FIG. 6. **Western blot analysis with immunodetection of the complete TER open reading frame in pET32a expressed in Rosetta (DE3).** Detection was carried out with anti-His antibody (monoclonal mouse IgG, Novagen). Anti-mouse secondary antibody horseradish peroxidase conjugate from goat was used and the 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.

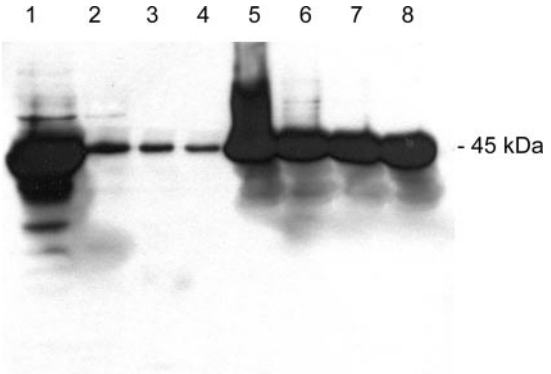

FIG. 7. **Western blot analysis with immunodetection of the 405-aa C-terminal part of the TER cDNA clone in pET28a expressed in BL21(DE3).** Detection was carried out with anti-His antibody (monoclonal mouse IgG, Novagen). Anti-mouse secondary antibody horseradish peroxidase conjugate from goat was used 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.

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 a novel protein family widespread among genomes. The K_m 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 k_m for *trans*-hexenoyl-CoA was 1.3

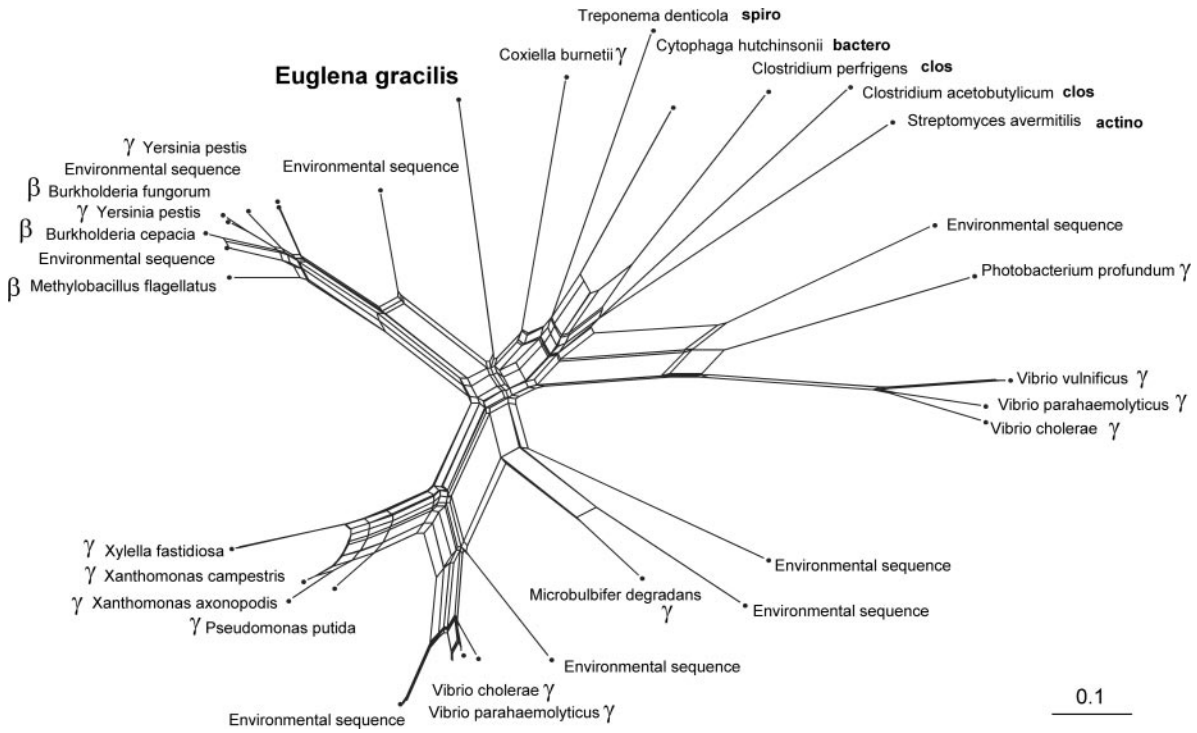

FIG. 8. **Pattern of *Euglena* TER sequence similarity.** NeighborNet planar graph of protein LogDet distances among TER homologues are shown. Splits (bifurcations, branches) in the data are indicated as a series of parallel lines. The *scale bar* at the *lower right side* indicates estimated substitutions per site. Accession numbers are given in clockwise order and are as follows: *Methylobacillus flagellatus* AADX0100001; Environmental sequence (EnvSeq) AACY01005767; *Burkholderia cepacia* AAEH01000008; *Yersinia pestis* AL590842; *Burkholderia fungorum* AAAJ02000008; EnvSeq AACY011006966; *Yersinia pestis* AE017142, EnvSeq AACY01755659; *E. gracilis* TER, *Coxiella burnetii* AE016960; *Treponema denticola* AE017248; *Cytophaga hutchinsonii* AADD03000005; *Clostridium perfrigens* AP003185; *Clostridium acetobutylicum* AE001437; *Streptomyces avermitilis* AB070934; EnvSeq AACY01025655; *Photobacterium profundum* CR378671; *Vibrio vulnificus* AE016808; *Vibrio parahemeolyticus* AP005087; *Vibrio cholerae* AE004406; EnvSeq AACY01756806; EnvSeq AACY01078371; *Microbulbifer degradans* AABI03000010; EnvSeq AACY01012997; *Vibrio cholerae* AE004251; *Vibrio parahemeolyticus* AP005077; EnvSeq AACY01098900; *Pseudomonas putida* AE016791; *Xanthomonas axonopodis* AE011637; *Xanthomonas campestris* AE012106; *Xylella fastidiosa* AE004005. Abreviations are as follows: β and γ, proteobacteria; *actino*, actinobacteria; *bactero*, bacteroides; *clos*, clostridia; *spiro*, spirochaetes.

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 iPSORT (35) and only a few transit peptides of *Euglena* 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 vivo*. However, the 135-aa N-terminal ORF is neither a cloning artifact, because it was observed in independent cDNAs (Fig. 4), nor is it essential for the activity of the enzyme, but it appears to interfere with the activity of the enzyme, because the construct possessing the 135-aa N-terminal ORF is soluble (Fig. 6) but inactive, whereas constructs lacking it are of the same M_r as the native enzyme size and are active (Fig. 7).

A putative NAD(P)H binding site (G*XXX*G*X*G) typical for the family of short-chain dehydrogenases/reductases (37) was found in *Euglena* TER at G^{190}CSTGYG^{196} (Fig. 4). A potential FAD binding site (G*X*G*XX*G) (38, 39) was identified in residues 515–520 (Fig. 4) near the C terminus of the sequence. FAD dependence of TER was suggested by Inui *et al.* (31) and confirmed here: lack of FAD in buffers during purification of TER from *Euglena* led to complete loss of enzyme activity. However, the recombinant enzyme expressed 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 recominant 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 Y*XXX*K (37, 40, 41), and some show a modified motif with $YX_{3-7}K$ (42, 43). TER from *Euglena* possesses the motif YX_6K at residues 231–238 and YX_5K at residues 279–285 (Fig. 4). In general, enoyl thioester reductases constitute a heterologues group 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* (Mrf1p) were identified that belong to the family of medium chain dehydrogenases/reductases (45, 46).

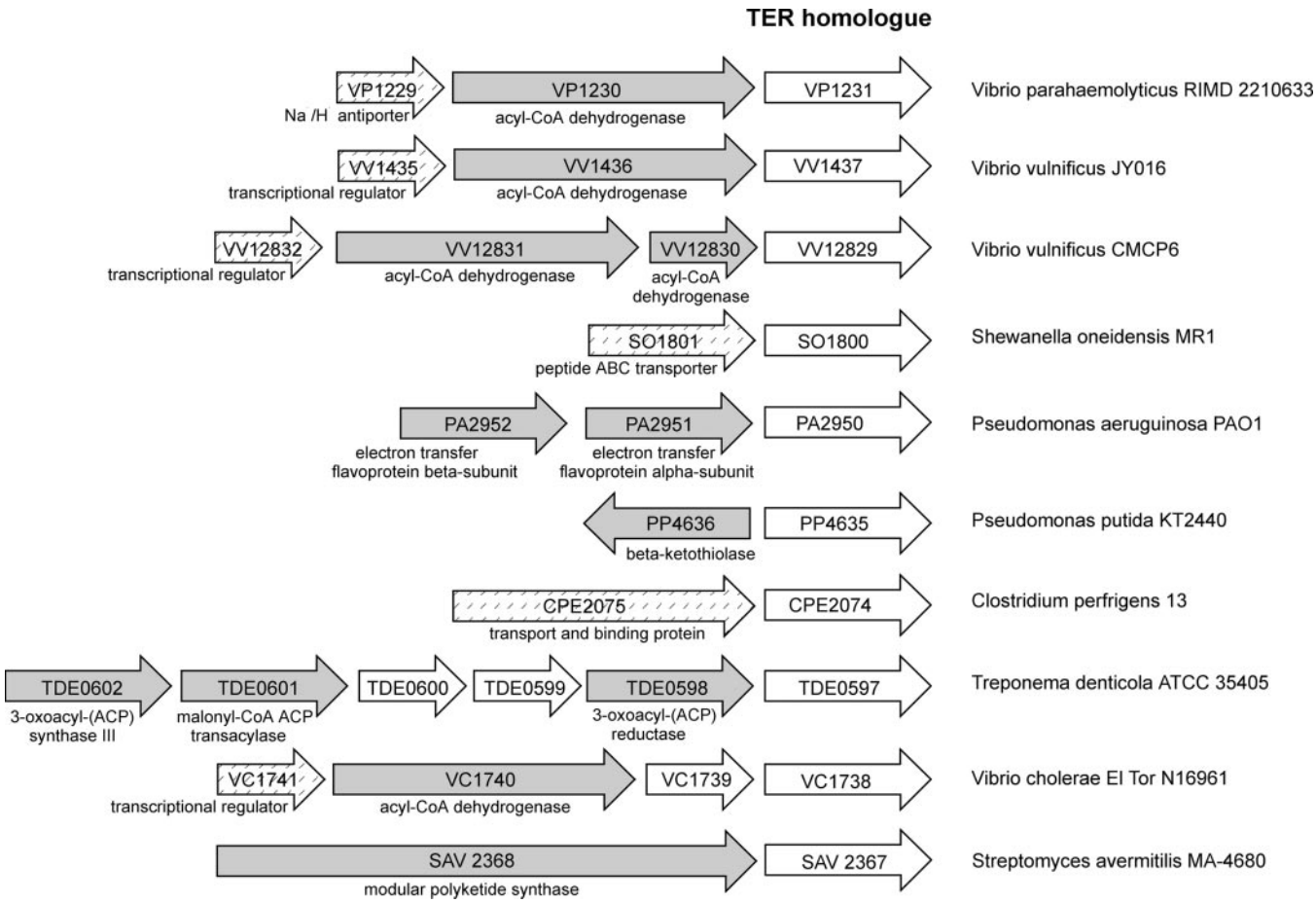

FIG. 9. **Operon analysis of homologues of *Euglena* TER via the CMR resource at TIGR.** *Open arrows* indicate hypothetical reading frames, *shadowed arrows* show ORFs involved in fatty acid biosynthetic pathways, and *hatched arrows* indicate miscellaneous functions.

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 collinus* 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. collinus* 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 γ-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-hydroxylacyl

thioester, (iii) dehydration to form the double bond in the resulting enoyl thioester, and (iv) reduction of the double bond to form a 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 C18 fatty acids to C20 and longer, generally operate with CoA esters (64). In mitochondria and peroxisomes, β-oxidation operates through steps iv to i with the CoA esters, whereby the oxidative step (iv) is physiologically irreversible, because it is finally linked to oxygen reduction.

In principle, mitochondrial fatty acid synthesis supporting wax ester fermentation in *Euglena* could occur: (*a*) via the reversible enzymatic steps of β-oxidation under the participation of TER, (*b*) via a yet undescribed CoA-dependent FAS system, or (*c*) via polyketide synthesis. TER catalyzes the step corresponding to step iv above with the CoA ester, reducing crotonyl-CoA to butyryl-CoA. We found no TER activity in the reverse reaction using butyryl-CoA as substrate with either NAD^+ or $NADP^+$. The recombinant TER enzyme showed the highest activity with crotonyl-CoA but also reduces C6 enoyl-CoAs and thus relates in terms of substrate preference to enoyl-Co reductase I described previously by Inui *et al.* (15).

The synthesis of fatty acids, in particular long-chain polyunsaturated fatty acids, via polyketide synthesis has been recently shown both for the prokaryote *Shewanella* and the eukaryote *Schizochytrium* (59, 65). The sequenced genomes of *Shewanella frigidmarina* and *Shewanella oneidensis* do possess TER homologues, but no homologues for TER could be identified from any eukaryote other than *Euglena.* The apicomplexan protist *Cryptosporidium parvum*, which possesses the same unusual pyruvate:$NADP^+$ oxidoreductase as *Euglena* (8), was recently found to encode a polyketide synthase (66), and various groups of dinoflagellates synthesize large and complex polyketides (67). However, polyketides are not specific to eukaryotic groups that possess plastids (or, in the case of *Cryptospridium*, have secondarily lost plastids), because various fungi also synthesize polyketides and possess polyketide synthases genes (68, 69).

Purification and cloning of TER, the key enzyme of mitochondrial fatty acid biosynthesis in *Euglena* defines a new family of genes in prokaryotes, homologues of which are found in association with genes from both polyketide biosynthesis and fatty acid biosynthesis in prokaryotes. Although our findings shed light on several dozen sequences from sequenced genomes, they provide disappointingly few clear hints as to the nature of the remaining enzymes involved in mitochondrial fatty acid biosynthesis in *Euglena*.

Evolutionary Considerations—Euglena mitochondrial TER is clearly an acquisition from a eubacterial donor, but whether that donor was the ancestral mitochondrion or whether *Euglena* TER represents an outright acquisition outside the context of mitochondrial origins (or possibly plastid origins) is not yet clear. Previous studies have revealed similar phylogenetic pictures as we observe for TER (Fig. 8), a lone eukaryotic sequence branching deeply within a group of eubacterial homologues. Such results are commonly interpreted as evidence for lateral gene transfer, sometimes with the implication that acquisition might relate to adaptations to anaerobic lifestyle (70). However, as more eukaryotic lineages become sampled, it often turns out that additional disparate eukaryotic groups are found to possess the same unusual gene, suggesting an acquisition by the common eukaryotic ancestor (71–73), whereby the view that the mitochondrion was the donor would then be the simpler, hence preferable, null hypothesis (74). It is noteworthy that *Euglena* TER does not group specifically with any of the sequences sampled, but rather seems to reflect an ancient acquisition in the *Euglena* lineage rather than a recent one (Fig. 8) and that TER homologues are most widespread among proteobacteria in the current prokaryotic sample, which would be generally consistent with mitochondrial origin. Searching genome data bases, we were unable to find other eukaryotes that possess bona fide TER homologues. If TER comes from the mitochondrion, then additional eukaryotic lineages should eventually be found that harbor the same gene.

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