Enoate Reductases of Clostridia
CLONING, SEQUENCING, AND EXPRESSION

Felix Rohdich‡, Anja Wiese, Richard Feicht, Helmut Simon, and Adelbert Bacher

From the Institut für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstraße 4, D-85747 Garching, Germany

The enr genes specifying enoate reductases of Clostridium tyrobutyricum and Clostridium thermoaceticum were cloned and sequenced. Sequence comparison shows that enoate reductases are similar to a family of flavoproteins comprising 2,4-dienoyl-coenzyme A reductase from Escherichia coli and old yellow enzyme from yeast. The C. thermoaceticum enr gene product was expressed in recombinant Escherichia coli cells growing under anaerobic conditions. The recombinant enzyme was purified and characterized.

Enoate reductases (EC 1.3.1.31) from Clostridium tyrobutyricum and Clostridium kluyveri catalyze the NADH-dependent reduction of carbon-carbon double bonds of nonactivated 2-enoates as well as of α,β-unsaturated aldehydes, cyclic ketones, and methylketones (Fig. 1) (for review see Refs. 1 and 2). Enzyme-catalyzed reactions similar to those catalyzed by enoate reductase are shown in Fig. 1.

Enoate reductases are characterized by high stereospecificity, strict regioselectivity, and rather broad substrate specificity. Reduced methylviologen can serve as an effective electron donor instead of NADH. Using this artificial electron transporter, enzymatic reductions can be carried out in electrochemical cells (2, 3). Selective dehydrogenation of saturated aldehydes can be performed using artificial electron acceptors (4). Thus, the C. tyrobutyricum enzyme is a useful reagent for the preparation of many chiral compounds, and, in particular, of chirally deuterium-substituted compounds (3–10).

Enoate reductases have been found in numerous Clostridia including some proteolytic species (11). An antiserum against enoate reductase from Clostridium tyrobutyricum cross-reacted with a protein of the thermophilic Clostridium thermoaceticum, but enoate reductase activity could not be detected in crude extracts of C. thermoaceticum using NADH as electron donor (12).

In Clostridium sporogenes, an enoate reductase appears to be involved in the reductive branch of the Stickland fermentation of amino acids. The reduction of 2-enoates may be coupled with ATP formation (1, 13).

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The nucleotide and amino acid sequence(s) of the enr genes of C. tyrobutyricum and C. thermoaceticum and partial gene of C. kluyveri have been submitted to the GenBank™/EMBL Data Bank with accession numbers Y09860, Y16136, and Y16137.

‡ To whom correspondence should be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13364; Fax: 49-89-289-13363; E-mail: felix.rohdich@ch.tum.de.

Fig. 1. Enzymes catalyzing the reduction of unsaturated acids and aldehydes. A, enoate reductase, EC 1.3.1.31; X = OH; H, Me (for the variability of R, R', and R", see Refs. 1–3); B, 2-enoyl-CoA reductase, EC 1.3.1.6, enoyl/acylcarrier protein) reductase, EC 1.3.1.9/1.3.1.10; (NADH/NADPH), butyryl-CoA dehydrogenase, EC 1.3.99.2; C, 2,4-dienoyl-CoA reductase of E. coli, EC 1.3.1.34. It should be noted that the hydrogen addition catalyzed by enoate reductases and enoyl-CoA reductases are both trans but stereochemically opposite.

Enoate reductase from C. tyrobutyricum is a 940-kDa homodecamer of 75-kDa subunits (14). Each subunit comprises an Fe₄S₄ cluster as well as one molecule each of FMN and FAD (1, 14, 15). This paper reports the cloning and sequencing of enr genes specifying enoate reductase of C. tyrobutyricum, C. thermoaceticum, and C. kluyveri. The C. thermoaceticum gene was expressed in enzymatically active form in recombinant Escherichia coli.

EXPERIMENTAL PROCEDURES

Materials—Immobilon™ P transfer membranes were obtained from Millipore (Eschborn, Germany). Hybond N+ membranes Hyperfilm ECL™ films, ECL™ direct nucleic acid labeling and detection system, and restriction enzymes were obtained from Amersham Pharmacia Biotech (Freiburg, Germany). T4 Ligase was obtained from Life Technologies, Inc. (Eggenstein, Germany), Goldstar Taq polymerase was from Eurogentec (Seraing, Belgium), Proteinase K from Sigma (Deisenhofen, Germany), RNase A from Macherey-Nagel (Düren, Germany), and DNase I from Roche Molecular Biochemicals (Mannheim, Germany). Anti-rabbit IgG (Fc) alkaline phosphatase conjugate was purchased from Promega (Madison, WI). 5-Bromo-4-chloro-indolyl-3-phosphate and nitro blue tetrazolium chloride were from Sigma (Deisenhofen, Germany), RNase A from Macherey-Nagel (Düren, Germany), and DNase I from Roche Molecular Biochemicals (Mannheim, Germany). Anti-rabbit IgG (Fc) alkaline phosphatase conjugate was purchased from Promega (Madison, WI). 5-Bromo-4-chloro-indolyl-3-phosphate and nitro blue tetrazolium chloride were from Sigma (Deisenhofen, Germany), Isopropyl-1-thio-β-D-galactopyranoside was from Eurogentec (Seraing, Belgium), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside was purchased from Bachem Biochemica GmbH (Heidelberg, Germany). NADH was purchased from Biomol (Hamburg, Germany). (E)-2-Methyl-2-butenoate was obtained from EGA (Steinheim, Germany). Oligonucleotides were custom synthesized by MWG-Biotech (Ebers-
treated with proteinase K and RNase A (19). Sodium chloride was subsequently with one volume of phenol/chloroform/isoamyl alcohol and maintained as described previously (16, 17).

Isolation of Clostridium DNA—Overnight cultures of Clostridium strains were lysed with sodium dodecyl sulfate (SDS). The lysates were incubated for 15 min at 94 °C and for 15 min at 60 °C. The mixture was extracted with one volume of chloroform/isoamyl alcohol (24:1) and isopropyl alcohol precipitation (19).

DNA Sequencing—DNA sequencing was performed by the automated dideoxy chain termination method (20) using a 377 Prism auto- 

Primer Amplifications—PCR reactions with degenerate primers contained 2–5 ng of DNA template and 25 pmol of each respective primer. Reactions with nondegenerate primers contained 2–5 ng of DNA template and 25 pmol of each respective primer. Other components were used as recommended in the Goldstar PCR System from Perkin-Elmer thermocycler (Norwalk, CT) at 94 °C (1 min), 50 °C (1 min), 72 °C (1–3 min) for 25–30 cycles. Purification of DNA Fragments—DNA fragments were isolated using the GeneClean II kit from Bio 101 Inc. (La Jolla, CA) or the PCR purification kit from Qiagen (Hilden, Germany).

DNA Minilibraries—Chromosomal DNA of C. tyrobutyricum was digested with EcoRI. The fragments were subjected to agarose gel electrophoresis. DNA fractions of the desired length were isolated from the gel and were ligated into EcoRI-digested plasmid pUC18 DNA. Cells of electocompetent E. coli XL1-Blue (21) were transformed with the ligation mixtures and were plated on agar plates containing ampicillin (180 μg/ml–1), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (50 μg/ml–1), or isopropyl-1-thio-β-D-galactopyranoside (150 μg/ml–1) as appropriate.

Isolation of Clostridium DNA—Overnight cultures of Clostridium strains were lysed with sodium dodecyl sulfate (SDS). The lysates were treated with proteinase K and RNase A (19). Sodium chloride was added to a final concentration of 0.7 M, and cetyltrimethylammonium bromide was added to a final concentration of 1.25% (v/v). The mixture was extracted with one volume of chloroform/isomyl alcohol (24:1) and subsequently with one volume of phenol/chloroform/isomyl alcohol (25:24:1). High molecular weight DNA was obtained from the supernatant by isopropyl alcohol precipitation (19).

Plasmid DNA Isolation—Plasmid DNA was isolated from the plasmid DNA isolation kit from Qiagen (Hilden, Germany).

DNA Sequencing—DNA sequencing was performed by the automated dideoxy chain termination method (20) using a 377 Prism automated DNA sequencer from Perkin-Elmer Life Sciences (Norwalk, CT).

PCR Amplifications—PCR reactions with degenerate primers contained 50–100 ng of DNA template and 500 pmol of each respective primer. Reactions with nondegenerate primers contained 2–5 ng of DNA template and 25 pmol of each respective primer. Other components were used as recommended in the Goldstar Taq-Polymerase kit from Eurogentec (Seraing, Belgium). Temperatures were cycled with the GeneAmp PCR System from Perkin-Elmer thermocycler (Norwalk, CT) at 94 °C (1 min), 50 °C (1 min), 72 °C (1–3 min) for 25–30 cycles.

Preparation of a Vector for Direct Cloning of PCR Fragments—The plasmid vector pBluescript II linearized with EcoRV and 3'-tailed as described by Mead et al. (22) was designated pBlue-EV-t.

Southern Blotting—Restriction fragments of chromosomal DNA were separated by agarose gel electrophoresis and transferred to Hybond N+ membranes by vacuum blotting. DNA/DNA hybridization was carried out as described in the ECL™ direct nucleic acid labeling and detection

b DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

a American type culture collection, patent deposit number.

| Primer | Sequence | Residue location on enoate reductase from C. tyrobutyricum | DNA sequence | Protein sequence |
|--------|----------|----------------------------------------------------------|---------------|-----------------|
| Eno1   | 5’-TTYGARGTNATHAAATTHGG-3’ | F E V L K I G | 315 → 334 | 7 → 13 |
| Eno2   | 5’-TCRTANARGTGCNACRTG-3’ | E Y L T V V H | 1576 ← 1594 | 427 ← 433 |
| Eno3   | 5’-NSWACYYTCTTYYCTC-3’ | S V K D K E M | 1817 ← 1835 | 507 ← 513 |
| Eno4   | 5’-ATGCCGNTATGGGNTTGGG-3’ | M A V M G A F G | 363 ← 385 | 27 ← 34 |
| Eno5   | 5’-GTCAGTTAGCTGATCTGAAAC-3’ | D P V S G P V | 1523 ← 1643 | 443 ← 449 |
| Eno6   | 5’-CTGAAATGTCATGCATGACGG-3’ | T E R V H A Y G | 577 ← 598 | 94 ← 101 |

\[ \text{a The primers were designed from peptide fragments and on the basis of sequence similarity of enoate reductase from C. tyrobutyricum with the enzymes listed in Table VII.} \]

\[ \text{b The primers Eno5 and Eno6 were designed on the basis of the DNA sequence of the partial enr gene of C. tyrobutyricum from the plasmid pBlue-Ev3/4. The corresponding amino acid sequences of the oligonucleotides are written in bold letters (IUPAC one-letter code). Locations and orientation of these oligonucleotides and corresponding peptide fragments on the DNA, respectively, protein sequence of enoate reductase from C. tyrobutyricum are indicated with arrows.} \]
system from Amersham Pharmacia Biotech (Freiburg, Germany).

**Cloning of the enr Gene of C. tyrobutyricum**—PCR experiments with
dNA were performed using degenerate oligonucleotides (Table II) in various combinations. An experiment using the oligonucleotides Eno3 and Eno4 afforded an amplificate of 1.4 kb which was ligated into plasmid pBLUE-EV-t. The ligation mixture was transformed into *E. coli* XL1-Blue cells. The resulting plasmid pBlueEno3/4 served as template in a PCR reaction using the oligonucleotides Eno5 and Eno6 as primers (Table II). The amplificate was labeled with the ECL™ direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) and was used as probe for Southern blot analysis of *Eco* RI-digested C. tyrobutyricum DNA where it was shown to hybridize with fragments of 1.3, respectively, 3.7 kbp. These fragments were cloned by colony blotting from minilibraries of *Eco* RI fragments affording the plasmids pUC18E1.3kb and pUC18E3.7kb as shown in Fig. 2.

**Cloning of the enr Gene of C. thermoaceticum**—PCR experiments were performed with *C. thermoaceticum* DNA as template using consensus primers shown in Table III in various combinations. The primer pair ERTERM2 and ER5 afforded an amplificate of 1.6 kbp (Fig. 3). This DNA segment was cloned and sequenced into the plasmid pBlue-ERNTERM2/ER5 yielding the plasmid pBlue-ERNTERM2/ER5 which was shown by sequencing to contain an insert of 1603 bp.

To extend that sequence in the 3′ direction, a PCR reaction was performed using chromosomal DNA as template and the oligonucleotides Th-ER2a and the degenerate “anchor-primer” (Life Technologies, Inc.) as primers. The resulting 0.5-kb amplificate served as template in a second PCR amplification using the oligonucleotides Th-ER2 and “UAP-primer” (Life Technologies, Inc.) as primers (Fig. 3). The resulting DNA fragment was cloned and sequenced.

To extend the DNA sequence of the open reading frame in the 5′ direction, chromosomal DNA was digested with *Aat* II, *Eco* RI, or *Mfe* I. The DNA fragments resulting from each experiment were ligated to the double strand DNA obtained by hybridization of the oligonucleotides LIG1 and LIG2 (see above). The ligation mixtures were used as template for PCR amplifications using the oligonucleotides LIG1 and Th-ER1a. A 0.3-kb amplificate obtained from the experiment per-

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**TABLE III**

Degenerate consensus primers used for cloning of enr genes of *C. thermoaceticum* and *C. kluyveri*

| Primer* | Sequence | Residue location on enoate reductase from *C. tyrobutyricum* |
|---------|----------|----------------------------------------------------------|
| ERTERM1 | 5′-ATG AAR AAY AER WSN YTN TTY GAR CC-3′ | 297 → 322 |
| ERTERM2 | 5′-TTY GAR CCN ATH AAR ATH GG-3′ | 315 → 334 |
| ERTERM3 | 5′-GYN AAR AAY AAR ATH WSN ATG-3′ | 344 → 365 |
| ERTERM4 | 5′-AAR ATH WSN ATG GCN CCN ATG-3′ | 354 → 374 |
| ER1     | 5′-GAY RAR TAY GGN GGN GA-3′ | 882 → 898 |
| ER2     | 5′-GN CCN TGY ATH GGN TGY CA-3′ | 1381 → 1399 |
| ER4     | 5′-CA NCC NAC NAR NCC NCC NCC-3′ | 1893 ← 1912 |
| ER5     | 5′-GT YTC RCA NCC NAC NAR NCC NCC-3′ | 1899 ← 1918 |

*The primers were designed from the DNA sequence of the *enr* gene of *C. tyrobutyricum*.
formed with AatII-digested DNA was used as template in a second PCR amplification using the oligonucleotides LIG1 and Th-ERR1 (Fig. 3). The resulting DNA fragment was cloned and sequenced.

**Construction of an Expression Plasmid for the enr Gene of C. tyrobutyricum**—The enr gene was assembled from two DNA segments prepared as described below. Amplification of C. tyrobutyricum DNA with the primers EnoA and Th-ERY1 (Table V) afforded a 0.4-bp fragment. This fragment served as template for a second amplification using the primers kEcoRI and Th-ERY1 (Table V). The resulting fragment was digested with EcoRI and BamHI. Similarly, PCR amplification of C. thermoaceticaum DNA with the primers ER2BamII and EnoB (Table V) afforded a 1-kbp segment which was cloned and sequenced.

**Construction of an Expression Clone for the enr Gene of C. thermoaceticaum**—A major part of the C. kluveri enr gene was obtained by the same approach as described above for the C. thermoaceticaum gene. Amplification of C. kluveri DNA with primers ER1 and ER4 (Table III) afforded a 1-kbp segment which was cloned and sequenced. Sequence extension in 5' direction was performed by ligation mediated two consecutive nested PCR experiments using the oligonucleotides LIG1, K-ER2 and K-ERR2 (Table IV), as described above. The resulting 0.4-kb DNA fragment was cloned and sequenced.

**Estimation of Protein Concentration**—Protein concentration was estimated using the method of Lowry et al. (25).

**Protein Sequencing**—Protein samples dissolved in 3% SDS containing 3% mercaptoethanol and 0.1% bromphenol blue were subjected to SDS-PAGE (26). Western Blotting—Enoate reductase from C. tyrobutyricum was treated with 2-mercaptoethanol, subsequently alkylated with 4-vinylpyridine and treated with cyanogen bromide (25). The resulting peptides were separated by high performance liquid chromatography and N-terminal sequences were obtained by automated Edman degradation (25).

**Polyacrylamide Gel Electrophoresis—SDS-PAGE** was performed with the SE 250 Mighty small II electrophoresis system from Amerham Pharmacia Biotech (Freiburg, Germany) at a constant current of 20 mA per gel, using 5% acrylamide stacking gels and 15% acrylamide concentration of 0.1M. The reaction was monitored photometrically (25 nm).

**Peptide fragment N-terminal sequence**

1 (N terminus) MKNKSLFEVIIKGVVXXIXMAMAGF
2 (M)DAIIXSNXICGNC
3 (M)GLLARPLISDAY
4 (M)AVLRGHTYLYESTD
5 (M)EKDVSTAVDILNCT
6 (M)LEDIXNYYNIVX
7 (M)KQFHVFIEHEEIVT
8 (M)KELQK(X)G
9 (M)KELQK(X)G
10 (M)KELQK(X)G

**Oligopeptide fragments of enoate reductase of C. tyrobutyricum obtained after cyanogen bromide treatment**

**TABLE V**

| Peptide fragment | N-terminal sequence |
|------------------|---------------------|
| 1                | MKNKSLFEVIIKGVVXXIXMAMAGF |
| 2                | (M)DAIIXSNXICGNC |
| 3                | (M)GLLARPLISDAY |
| 4                | (M)AVLRGHTYLYESTD |
| 5                | (M)EKDVSTAVDILNCT |
| 6                | (M)LEDIXNYYNIVX |
| 7                | (M)KQFHVFIEHEEIVT |
| 8                | (M)KELQK(X)G |
| 9                | (M)KELQK(X)G |
| 10               | (M)KELQK(X)G |

**Oligopeptide fragments of enoate reductase of C. tyrobutyricum**

**TABLE VI**

| Peptide fragment | N-terminal sequence |
|------------------|---------------------|
| 1                | MKNKSLFEVIIKGVVXXIXMAMAGF |
| 2                | (M)DAIIXSNXICGNC |
| 3                | (M)GLLARPLISDAY |
| 4                | (M)AVLRGHTYLYESTD |
| 5                | (M)EKDVSTAVDILNCT |
| 6                | (M)LEDIXNYYNIVX |
| 7                | (M)KQFHVFIEHEEIVT |
| 8                | (M)KELQK(X)G |
| 9                | (M)KELQK(X)G |
| 10               | (M)KELQK(X)G |

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first antibody. Anti-rabbit IgG (Fc) conjugated to alkaline phosphatase was used as second antibody. Blots were developed with 5-bromo-4-chloroindol-3- phosphate in the presence of the reduct mediator nitroblue tetrazolium (29).

**Purification of Reconstituent Enolate Reductase from** *C. thermoacetica*—All buffers used for column chromatography contained 1 mM dithiothreitol, 1 mM EDTA, and 250 mM sucrose. Frozen cell mass (2 g) was thawed in 10 ml of 100 mM sodium phosphate, pH 7.0, under an atmosphere of argon. Lysosome (4 mg ml\(^{-1}\)) and DNase I (0.4 mg ml\(^{-1}\)) were added. The suspension was incubated for 30 min at 37 °C, ultrasonically treated 3 times for 15 s at 4 °C with a Branson Sonifier 250 (Branson SONIC Power Co., Danbury, CT), and centrifuged (45 min, 17,000 rpm, SS34 rotor, Sorvall RC-5B Plus centrifuge, DuPont Instruments). The supernatant was diluted 1:5 with water and loaded on a Q-Sepharose FF column (Amersham Pharmacia Biotech, Freiburg, Germany, 2 cm × 12 cm) which had been equilibrated with 20 mM sodium phosphate, pH 7.4. The column was developed with a linear gradient of 0–2 M KCl in 20 mM sodium phosphate. Enzyme containing fractions were combined, concentrated 1:1 by ultrafiltration (50 kDa) and diluted 1:1 with 20 mM sodium phosphate, pH 7.4. The solution was loaded on a Mono Q HR column (Amersham Pharmacia Biotech, 0.5 cm × 5 cm) which had been equilibrated with 20 mM sodium phosphate, pH 7.4. The column was developed with a linear gradient of 0–2 M KCl in 20 mM sodium phosphate. Enzyme containing fractions were combined and applied to a Superdex G 200 column (Amersham Pharmacia Biotech, 60 cm) which was developed with 20 mM sodium phosphate, pH 7.4, containing 50 mM NaCl. The flow rate was 4 ml min\(^{-1}\). Fractions were combined and concentrated by ultrafiltration.

**RESULTS**

**Cloning of the enr Gene of** *C. tyrobutyricum*—Enolate reductase of *C. tyrobutyricum* (14) was treated with cyanogen bromide, and the resulting peptides were separated and analyzed by Edman degradation. Partial amino acid sequences of the N terminus and of 5 peptide fragments (Table VI) suggested sequence similarity between enolate reductase and several flavin oxidoreductases shown in Table VII. Degenerate oligonucleotides (Table II) were designed on the basis of the partial sequences. PCR amplification using oligonucleotides Eno3 and Eno4 as primers and chromosomal DNA of *C. tyrobutyricum* as template afforded an amplificate of ~1.4 kb (Fig. 2) which was cloned into the pBluescript SKII (bacterium W3A1) (14).

**Enzymes**

**Enolate reductase**

| Enzyme                          | EC      | Microorganism          | Gene | Accession number | Ref.      |
|---------------------------------|---------|------------------------|------|------------------|----------|
| Enolate reductase               | 1.3.1.31| *C. tyrobutyricum*     | *enr*| Y09960           | This study|
|                                 |         | *C. thermoacetica*     |      | Y16136           | This study|
|                                 |         | *C. kluyveri*          |      | Y16137           | Thiastudy|
| NADH:flavin oxidoreductase      | 1.6.99.3| *Thermodesulbacter brockii* |    | X67220          | 37       |
| NADH:acceptor oxidoreductase    | 1.3.1.31| *Rhodobacter capsulatus* |    | 2650172         | 33       |
| NADH oxidase                    | 1.6.99.1| *Arsenophorus fulgidus* | nox1B | X98353          | 46       |
| NADPH dehydrogenase             | 1.3.1.31| *Acinetobacter calcoaceticus* |  | X60233         | 35       |
| NADPH dehydrogenase (old yellow enzyme) | 1.3.1.31| *Serratia marcescens* | y1e | X53957          | 47       |
| NADPH dehydrogenase (old yellow enzyme) | 1.3.1.31| *Eubacterium sp. strain* | baiC | M36292         | 48       |
| 2,4-Diencyl-CoA reductase        | 1.3.1.34| *Escherichia coli*     | fhdH | U93405          |          |

*a* Fragment.

*b* Identified by sequence similarity.

*GenBank* accession number.

For this purpose, *C. tyrobutyricum* DNA was digested with EcoRI and analyzed by Southern blotting. A probe derived from the known gene segment recognized two fragments of 1.3 and 3.7 kbp. Minilibraries containing fragments of these respective lengths were then constructed and screened by colony hybridization affording two plasmids with 1.3- and 3.7-kbp inserts which were sequenced from both ends.

The resulting DNA sequence (*GenBank* accession number Y09960) comprised an open reading frame of 2001 bp preceded by a putative ribosomal binding site and followed by a putative palindromic terminator sequence (Fig. 4). The open reading frame predicted a protein of 667 amino acids with a molecular mass of 72.8 kDa in line with the relative mass of 73 kDa which had been reported earlier for enolate reductase of *C. tyrobutyricum* (14).

**Cloning of the enr Genes from** *C. thermoacetica* and *C. kluyveri*—Consensus PCR primers (Table III) were designed on the basis of sequence similarity between enolate reductase of *C. tyrobutyricum* and the enzymes shown in Table VII. Specifically, the oligonucleotides were designed to match highly conserved segments of the enolate reductase sequence as shown in Fig. 5.

The oligonucleotides were used as primers in PCR experiments with chromosomal DNA from various *Clostridia* as templates (Table I). A 1-kb fragment of the putative *enr* gene was obtained from *C. kluyveri* DNA using the primers ER1 and ER4, and a 1.6-kb fragment was obtained from *C. thermoacetica* DNA using the primers ERNTER2 and ER5. These fragments were cloned into the plasmid vector pBlue-EV-t and sequenced.

The partial open reading frame from *C. thermoacetica* was extended in both directions as described under “Experimental Procedures.” The resulting DNA segment of 2142 bp (*GenBank* accession number Y16136) comprised an open reading frame of 2001 bp predicting a peptide of 667 amino acid residues with a mass of 73.0 kDa. The partial *enr* gene of *C. kluyveri* was similarly extended as described under “Experimental Procedures,” but only a partial open reading frame of 1359 bp specifying 453 amino acid residues was obtained.

The predicted amino acid sequences of the enolate reductases from *C. tyrobutyricum* and *C. thermoacetica* and the amino acid sequence predicted by the partial open reading frame *C. kluyveri* are highly conserved. The sequences of the proteins from *C. thermoacetica* and *C. tyrobutyricum* show 59% identity. The identity between the partial enolate reductase from *C. kluyveri* and *C. tyrobutyricum* is 75%.

**Recombinant Expression of Enolate Reductase**—The open
reading frames of the *enr* genes of *C. tyrobutyricum*, respectively, *C. thermoaceticum*, were placed under control of the lac operator and a T5 promotor in the expression plasmid pNCO113. The expression plasmid constructs were designated pNCO-ERCTYR and pNCO-ERCTHERM, respectively. In *E. coli* cells growing under aerobic conditions, the recombinant *enr* genes of *C. tyrobutyricum* and *C. thermoaceticum* could be expressed as insoluble proteins accounting for about 1, respectively, 20% of total cell protein as shown by SDS-PAGE and Western blotting using an antiserum against enoate reductase from *C. tyrobutyricum* [Ref. 12, data not shown].

Under anaerobic conditions, the recombinant *E. coli* strain M15 carrying the plasmid pNCO-ERCTHERM afforded soluble, enzymatically active *C. thermoaceticum* enoate reductase.

**FIG. 4.** Nucleotide sequence and predicted amino acid sequence of the *enr* gene from *C. tyrobutyricum*. EcoRI restriction sites are underlined. The putative ribosome-binding site, start and stop codons are shown in bold letters and underlined. Nucleotide residues forming the stem-loop of a putative terminator sequence are shown in bold letters. Peptide sequences obtained by Edman degradation are boxed.

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Cell extracts had a specific enoate reductase activity of 0.1 μmol min⁻¹ mg⁻¹, pH 6.8, at 37 °C with (E)-2-methylbutenoate and NADH as substrates. This value was similar to the enoate reductase in cell extracts of *C. tyrobutyricum* (0.2 μmol min⁻¹ mg⁻¹).

The recombinant enzyme was purified to apparent homogeneity by column chromatography as described under “Experimental Procedures” (Fig. 6). It had a specific activity of 2.4 μmol min⁻¹ mg⁻¹. Gel filtration experiments indicated a relative mass larger than 0.6 MDa. The enzyme showed activity in the pH range from 5.0 to 9.0 with a maximum around pH 7.3. NADPH could not serve as substrate. The purified protein migrated as a single band on SDS-PAGE gels at 73 kDa (Fig. 6). These characteristics are similar to those of enoate reductase from *C. tyrobutyricum* (24).

The N-terminal sequence (34 amino acids) of purified enoate reductase from *C. thermoaceticum* was determined by automated Edman degradation. The amino acid sequence was identical to that predicted from the DNA sequence.

**DISCUSSION**

The amino acid sequences predicted by *enr* genes of *C. tyrobutyricum*, *C. kluyveri*, and *C. thermoaceticum* indicate that enoate reductases belong to a family of pyridine nucleotide-dependent flavoproteins (Table VII). An alignment of the sequences of enoate reductase from *C. tyrobutyricum* and the other protein sequences with the exception of old yellow enzyme from yeast is shown in Fig. 5. All sequences showed homology over their entire lengths (29–34% identity to enoate reductase of *C. tyrobutyricum*). A region with 4 conserved cysteine residues is present in all sequences. The consensus pattern C-(2X)-C-(2–3X)-C-(11–12X)-C is similar to Fe₄S₄ clusters.
Enzymes and microorganisms from Clostridia

| Enzyme                      | Microorganism                      | Subunit mass (kDa) | Quaternary structure | Cofactors per subunit | Electron donor |
|-----------------------------|------------------------------------|--------------------|----------------------|-----------------------|---------------|
| Enoate reductase            | C. tyrobutyricum                   | 72.8               | Dodecamer            | Fe,S₄, 1 FAD, 1 FMN   | NADH          |
| 2,4-Dienoyl-CoA reductase   | Escherichia coli                   | 72.6               | Trimer               | 1 FAD                 | NADPH         |
| NADH:flavin oxidoreductase  | Eubacterium sp. strain VPI 12708   | 72.0               | ND                   | 2 FAD                 | NADPH         |
| NADH:acceptor oxidoreductase| Thermotoga neapolitana brockii    | 71.2               | Hexamer              | Fe,S₄, 2 FAD          | NADPH         |
| Trimethylamine dehydrogenase| Methylophilus methylphilus        | 81.6               | Dimer                | Fe,S₄, 1 FMN, 1 ADP   | (CH₃)₃N       |
| Dimethylamine dehydrogenase | Hyphomicrobium X                   | 82.5               | Dimer                | Fe,S₄, 1 FMN, 1 ADP   | (CH₃)₃N       |

* ND, not determined.

**TABLE VIII** Properties of enzymes with sequence similarity to enoate reductase

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in ferredoxins and other iron-sulfur proteins (30, 31). X-ray crystallographic studies of trimethylamine dehydrogenase indicated that these 4 cysteines are ligands for the Fe₄S₄ cluster (32). With the exception of trimethylamine dehydrogenase and dimethylamine dehydrogenase, all sequences show two other well-conserved motifs (amino acid residues 404–431 and 529–546 in enoate reductase) with similarity to the binding sites for the ADP moiety of FAD and NAD(P)H in other flavoproteins (32–37).

Enoate reductases are similar to 2,4-dienoyl-CoA reductase from *E. coli* (38), but no similarity exists between enoate reductases and eukaryotic 2,4-dienoyl-CoA reductases (39). It should be noted that 2,4-dienoyl-CoA reductase of *E. coli* and eukaryotic organisms yield different products (40).

The N terminus of enoate reductases (residue 1–368) shares 28% identity (44% similarity) with old yellow enzyme from *Saccharomyces cerevisiae* (EC 1.6.99.1) which was recently shown to reduce 2-enals and methyl-3-enketones (41). The stereochemical course of the reaction is trans, opposite to that of enoate reductase. Old yellow enzyme does not contain iron-sulfur clusters. It catalyzes the transfer of the pro-R hydrogen from NADPH to the β-position of the substrate (42) in contrast to enoate reductase, which transfers the pro-S hydrogen (24).

Enoate reductases have no detectable similarity to enoyl-CoA reductases (43). This is in line with the different stereochemistry of the reaction products formed by the addition of hydrogen by enoate reductases and enoyl-CoA reductases (Fig. 1).

With the exception of trimethylamine dehydrogenase and dimethylamine dehydrogenase, the oxireductases with similarity to enoate reductases have subunit molecular masses the in the range of 71–73 kDa, but the subunit structure varies widely. All enzymes have been shown either directly or indirectly to have iron and acid-labile sulfide, and all contain at least one molecule of flavin. Reduced pyridine nucleotides served as electron donors with the exception of trimethylamine dehydrogenase and dimethylamine dehydrogenase, which have trimethylamine or dimethylamine as electron donors, respectively. These facts correspond well to the features across the amino acid sequences concerning the putative FeS-cluster-, FAD-, and NAD-binding sites (Table VIII). A comparison of the amino acid sequences of enoate reductases showed strong conservation between *C. tyrobutyricum* and *C. thermoaceticum* (59% identity), although the GC content of the DNA sequences from these microorganisms differs widely (30 and 54%, respectively (44, 45)).

Recombinant expression of enoate reductase from *C. tyrobutyricum* in aerobically grown *E. coli* host strains directed the formation of insoluble, inactive protein. The expression level was very low, so that the protein could only be detected on SDS-PAGE gels as a weak band and by Western blot analysis. This is likely because of the low GC content of the *C. tyrobutyricum* DNA (30%), which is also observed in the open reading frame of the *env* gene. This fact is reflected in a much different codon usage of *E. coli* and *C. tyrobutyricum*, which results in up to 90 so-called regulatory codons in the open reading frame (46).

Recombinant enoate reductase from *C. thermoaceticum* from aerobically grown *E. coli* host cells was insoluble and enzymatically inactive too, but was expressed to a level of about 20% of total cell protein (data not shown). This is likely due to the different GC content resulting in only 20 regulatory codons identified across the DNA sequence of the *env* gene from *C. thermoaceticum*. However, in anaerobically grown *E. coli* host cells recombinant enoate reductase was expressed in soluble and enzymatically active form. The recombinant enzyme was purified nearly to homogeneity and preliminary studies on characterization were performed indicating both differences and similarities in properties of the highly conserved enoate reductases from *C. tyrobutyricum* and *C. thermoaceticum*. The catalytic activity of recombinant enoate reductase from *C. thermoaceticum* (2.4 μmol min⁻¹ mg⁻¹) is similar to that of the enzyme isolated from wild type *C. tyrobutyricum* (10.6 μmol min⁻¹ mg⁻¹).

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