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Characterization of the *baiH* Gene Encoding a Bile Acid-Inducible NADH:Flavin Oxidoreductase from *Eubacterium* sp. Strain VPI 12708

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A cholate-inducible, NADH-dependent flavin oxidoreductase from the intestinal bacterium *Eubacterium* sp. strain VPI 12708 was purified 372-fold to apparent electrophoretic homogeneity. The subunit and native molecular weights were estimated to be 72,000 and 210,000, respectively, suggesting a homotrimeric organization. Three peaks of NADH:flavin oxidoreductase activity (forms I, II, and III) eluted from a DEAE–high-performance liquid chromatography column. Absorption spectra revealed that purified form III, but not form I, contained bound flavin, which dissociated during purification to generate form I. Enzyme activity was inhibited by sulfhydryl-reactive compounds, acriflavine, *o*-phenanthroline, and EDTA. Activity assays and Western blot (immunoblot) analysis confirmed that expression of the enzyme was cholate inducible. The first 25 N-terminal amino acid residues of purified NADH:flavin oxidoreductase were determined, and a corresponding oligonucleotide probe was synthesized for use in cloning of the associated gene, *baiH*. Restriction mapping, sequence data, and RNA blot analysis suggested that the *baiH* gene was located on a previously described, cholate-inducible operon ≥10 kb long. The *baiH* gene encoded a 72,006-Da polypeptide containing 661 amino acids. The deduced amino acid sequence of the *baiH* gene was homologous to that of NADH oxidase from *Thermoanaerobium brockii*, trimethylamine dehydrogenase from methylotrophic bacterium W3A1, Old Yellow Enzyme from *Saccharomyces carlsbergensis*, and the product of the *baiC* gene of *Eubacterium* sp. strain VPI 12708, located upstream from the *baiH* gene in the cholate-inducible operon. Alignment of these five sequences revealed potential ligands for an iron-sulfur cluster, a putative flavin adenine dinucleotide-binding domain, and two other well-conserved domains of unknown function.

Bile acids are steroids which aid in the degradation, solubilization, and absorption of lipids from the intestinal lumen. The primary bile acids in humans, cholate and chenodeoxycholate, are synthesized from cholesterol by the liver, conjugated to either glycine or taurine, and stored in the gallbladder. During digestion, these compounds are released into the proximal duodenum and are later reabsorbed by an active transport system in the distal ileum. Thereafter, they are returned, via the portal blood, to the liver where they are absorbed and reutilized. During their passage through the intestinal lumen, the bile acids are exposed to the indigenous intestinal microflora, many of which can modify the bile acids. These modifications include hydrolysis of the amide bond of conjugated bile acids, oxidation or reduction of hydroxy moieties, and the reductive 7-dehydroxylation of the primary bile acids (15, 19).

Quantitatively, the most physiologically important of the bile acid biotransformations is the 7α-dehydroxylation of the primary bile acids. It has been estimated that the colon contains 10^3 to 10^5 bacteria per ml capable of performing this reaction (12). All presently known strains which possess a stable bile acid 7α-dehydroxylation activity are anaerobic, gram-positive rods of the genera *Clostridium* and *Eubacterium*. Microbial 7α-dehydroxylation of the primary bile acids is the only known source of deoxycholate and lithocholate, which account for up to 25% of the total bile acid pool in humans (37). The secondary bile acids differ significantly from their parent compounds in their physicochemical properties and physiological effects. Such differences include decreased solubilization of lipids (6, 18), altered regulation of de novo cholesterol and bile acid biosyntheses (16, 17), increased cytotoxicity (41), and possible promotion of colon carcinogenesis (7). However, despite the importance of 7α-dehydroxylation to the host, the regulation of this pathway and its genetic distribution among the intestinal microflora are still incompletely understood.

The best-characterized intestinal isolate which possesses a 7α-dehydroxylation activity is *Eubacterium* sp. strain VPI 12708. This organism possesses a cholate-inducible enzyme system which can dehydroxylate both 7α- and 7β-bile acids (42–45). A mechanism for the 7α-dehydroxylation reaction, based on the isolation of bile acid intermediates and the conversion of these intermediates to 7α-dehydroxylated products, has been proposed (3, 8, 20). The 7α-hydroxy bile acid is first conjugated to ADP (8) or coenzyme A (28) and oxidized in two steps to yield a 3-oxo-4-cholenoic acid. This intermediate is then thought to undergo a *trans*-elimination of water across the C-6–C-7 bond to give a 3-oxo-4,6-choldienoic acid. Three successive reductive reactions then follow, resulting in the production of the corresponding 7α-dehydroxylated bile acid.

Cholate induces the biosynthesis of several new proteins in *Eubacterium* sp. strain VPI 12708 (42). Some of these cholate-inducible proteins have been purified to electrophoretic homogeneity, including the following: 19.5-kDa (29) and 45-kDa (46) proteins of unknown function, a 27-kDa putative 3α-hydroxysteroid dehydrogenase (9, 10), and, recently, a 58-kDa bile acid-coenzyme A ligase (28). The genes

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encoding these proteins are organized on three RNA transcripts. The 3α-hydroxysteroid dehydrogenase is encoded by three separate genes (9, 10, 14, 47). Two of these genes are short, monocistronic transcripts (10, 14), while the third is part of a large (>10-kb) polycistronic message (29, 46, 47). The latter transcript encodes six or more polypeptides and perhaps all of the remaining proteins involved in 7α-dehydroxylation (29).

The reductive steps of bile acid 7α-dehydroxylation in cell extracts of *Eubacterium* sp. strain VPI 12708 are stimulated by the addition of reduced flavins (45), suggesting the presence of an enzyme which provides reduced flavins for these steps. Indeed, Lipsky and Hylemon (25) detected a cholate-inducible, NADH-dependent flavin oxidoreductase (NADH:FOR) in cell extracts. The enzyme was partially purified (sixfold) and characterized. Several physical properties of the partially purified preparation, including pH optima, native molecular weight, and some preliminary kinetics, were determined. However, the enzyme could not be completely purified owing to inactivation. In this study, we report the successful purification of the cholate-inducible NADH:FOR from *Eubacterium* sp. strain VPI 12708, the cloning and sequencing of its associated gene (*baiH*), and a partial characterization of the enzyme.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Eubacterium* sp. strain VPI 12708 was cultured anaerobically and maintained as described previously (44) except that the growth medium was modified by replacing brain heart infusion broth with tryptic soy broth (30 g/liter). When desired, the cultures were induced with sodium cholate as described before (13). Strains of *Escherichia coli* were grown on Luria-Bertani broth or agar plates, supplemented with ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 250 μg/ml), or isopropylthio-β-galactoside (IPTG; 100 μg/ml) as appropriate (1).

Enzyme and protein assays. NADH:FOR activity was measured spectrophotometrically under aerobic conditions at 20°C by monitoring the oxidation of NADH at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) with a Shimadzu UV160U recording spectrophotometer. Assays contained the following in a final volume of 1 ml: sodium phosphate buffer (pH 6.8), 100 mM; NADH, 150 μM; flavin adenine dinucleotide (FAD), 150 μM; and an appropriate amount of enzyme. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH per min. Protein concentrations were determined by the dye-binding assay of Bradford, with bovine serum albumin as the standard (5). Elution of proteins during chromatographic steps was monitored spectrophotometrically at 280 nm by measuring the absorbance of individual fractions or by continuous monitoring with a Beckman 164 variable-wavelength detector.

Purification of NADH:FOR. All purification steps were performed aerobically. Soluble cell extracts were prepared by French pressure cell lysis and ultracentrifugation as described previously (13). Cell extract (62 ml; 2.6 g of protein) was dialyzed overnight (4°C) against 4 liters of buffer A (25 mM sodium phosphate [pH 6.8], 5% [vol/vol] glycerol, 1 mM dithiothreitol [DTT]). The dialyzed cell extract was loaded onto a column (2.5 by 20 cm) of DE-52 DEAE-cellulose (Whatman, Inc., Clifton, N.J.) equilibrated previously with buffer A at 4°C. The column was then washed with 50 ml of buffer A at a flow rate of 1.2 ml/min. Bound proteins were eluted with a 400-ml, linear, increasing NaCl gradient (0 to 500 mM in buffer A). Fractions were collected at 4-min intervals. Pooled DE-52 fractions were concentrated and desalted with a Centriprep 10 cartridge (Amicon, Danvers, Mass.). Excess salt was removed with three 10-ml aliquots of buffer A, with reconcentration between washes. The sample pH was then brought to 8.5 with NaOH, and the proteins were loaded onto a column (1 by 3 cm) of Reactive Red 120 agarose (Sigma Chemical Co., St. Louis, Mo.) which had been equilibrated with buffer B (25 mM sodium phosphate [pH 8.5], 5% [vol/vol] glycerol, 1 mM DTT) at 20°C. The column flowthrough was collected and saved for further purification. The loading eluate (unbound proteins) from the Reactive Red agarose column was collected, and the pH was adjusted to 7.0 by the addition of 0.1 N HCl. This solution was then loaded onto a Cibacron Blue A (Amicon) column (1.5 by 10 cm) which was equilibrated with buffer C (25 mM sodium phosphate buffer [pH 7.0], 5% [vol/vol] glycerol, 1 mM DTT) at 20°C, and the column eluate was reapplied to the column five more times. The column was then washed with 15 bed volumes of buffer C to remove any unbound protein. Bound proteins were eluted with buffer C containing 1 M KCl. All flow rates were about 1.0 ml/min, and fractions were collected at 4-min intervals. The pooled Cibacron Blue A fractions were concentrated and desalted with buffer C, using a Centriprep 10 cartridge as described above. The proteins were then loaded onto a preequilibrated Spherogel DEAE-3SW column (Beckman, Fullerton, Calif.) at a flow rate of 0.4 ml/min (20°C). The flow rate was increased to 0.85 ml/min when sample application was completed. Bound proteins were eluted with the following NaCl gradient in buffer C: 0 to 100 mM NaCl in 10 min, 100 to 300 mM NaCl in 80 min, and 300 to 500 mM NaCl in 10 min. Fractions were collected at 1-min intervals. Solid ammonium sulfate was added to the pooled DEAE–high-performance liquid chromatography (HPLC) fractions to a final concentration of 20% (wt/vol) at 4°C, and the pH was maintained at 7.0 by the addition of 1 N ammonium hydroxide. The protein sample was then loaded at a flow rate of 0.4 ml/min onto a Spherogel phenyl-5PW column (Beckman) which had been equilibrated with buffer C containing 20% (wt/vol) ammonium sulfate (20°C). After sample application, the flow rate was increased to 1.0 ml/min. Bound proteins were eluted with a 40-min, linear, decreasing ammonium sulfate gradient (20% [wt/vol] to 0%). The column was washed with 10 ml of buffer C without ammonium sulfate, and 1.0 ml of buffer C containing 10% (vol/vol) ethanol was then injected onto the column. Fractions were collected at 1.0-min intervals.

Electrophoresis conditions. Slab gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 20°C at a constant current of 1.5 mA/cm as described by Laemmli (23), except that 5% acrylamide stacking and 12% acrylamide separating gels were used. The gels were stained for protein with 0.2% (wt/vol) Coomassie R-250 in ethanol-acetic acid-water (25:7:68) and destained with the same solvent system lacking the Coomassie dye.

Native molecular weight determinations. A Sepharose CL-6B (Pharmacia LKB Biotechnology, Uppsala, Sweden) gel filtration column (2.5 by 75 cm) was equilibrated with buffer A containing 100 mM NaCl at a flow rate of 0.5 ml/min. The column was calibrated with blue dextran 2000, apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa), all from Sigma. A standard curve was obtained by plotting the log of molecular weight versus relative elution volumes (V_e/V_0), and the

native molecular weight of purified NADH:FOR was accordingly estimated from its V_e/V_0.

Generation of polyclonal antibodies. A male New Zealand White rabbit (2 kg) was obtained from Blue and Gray Rabbitry and housed in the Medical College of Virginia Animal Resources Facility. A 20-ml sample of preimmune blood was drawn from the ear, and the serum was collected and saved at −20°C. The animal was initially challenged by injecting 100 μg of purified protein, in Freund's complete adjuvant, intramuscularly into the thigh. The antibody titer was boosted with two subsequent injections of 100 μg of protein in Freund's incomplete adjuvant at 3-week intervals. Immune serum was collected 1 week after the second boost. After it was ascertained that the antibody titer was sufficiently high, the animals were sacrificed by exsanguination, and the immune serum was collected and stored at −20°C for further use.

Western blot (immunoblot) analysis. Protein samples were loaded and run on SDS–12% PAGE slab gels as described above, except that 0.01% (wt/vol) pyronin Y was added to each sample to act as a lane marker. Prestained molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.) were used for size determinations. The proteins were electrophoretically transferred to a nitrocellulose membrane, and immunoreactive bands were detected with the Bio-Rad Immun-Blot assay kit in accordance with the manufacturer's instructions. The rabbit polyclonal antisera (1:50 diluted) were utilized as the primary antibody, while goat anti-rabbit antiserum conjugated to horseradish peroxidase was used as the secondary antibody. Bound anti-rabbit antibodies were visualized by using 4-chloro-1-naphthol in the presence of 0.15% hydrogen peroxide.

Immunoinhibition studies. The purified proteins were placed in the standard assay buffer in the presence of different volumes of preimmune or immune serum. The mixtures were allowed to incubate for 15 min at 20°C. Enzymatic reactions were initiated by the addition of FAD and NADH and assayed spectrophotometrically as described above. Control reactions with no additions were also performed. No NADH:FOR activity was detected in the rabbit sera used.

Amino-terminal amino acid sequence determination. Purified NADH:FOR (approximately 1 nmol) was extensively dialyzed against HPLC-grade water (Alltech, Deerfield, Ill.) and concentrated to 200 μl with a 2-ml Centricon-10 concentrator (Amicon). The N-terminal amino acid sequence was determined at the University of Illinois, Urbana-Champaign, using an Applied Biosystems gas phase amino acid sequenator.

Oligonucleotide synthesis, purification, and labelling. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer at the Medical College of Virginia/Virginia Commonwealth University Nucleic Acids Core Facility, purified by thin-layer chromatography, and 5′-end labelled with [δ-^{32}P]ATP and T4-polynucleotide kinase as described previously (29).

Recombinant DNA methods. Total chromosomal DNA from *Eubacterium* sp. strain VPI 12708 was purified by the method of Marmur (30). Total RNA from this organism was purified as described previously (10). DNA from lambda gt11 isolates was purified from clarified lysates (1). Plasmid DNA was isolated by an alkaline lysis procedure (1). Single-stranded DNA from M13 phage subclones was prepared as described before (1). DNA from agarose gels was extracted with GeneClean (Bio 101 Inc., La Jolla, Calif.).

Restriction digestions, ligations, and other enzymatic manipulations were performed as described previously (1). DNA-DNA hybridizations were carried out after agarose gel electrophoresis of restriction fragments either directly in the dried gels or after alkaline transfer of the restriction fragments to GeneScreen nylon membranes (DuPont NEN Research Products, Boston, Mass.) (1). Screening of bacteriophage plaques and bacterial colonies was done with in situ hybridization as described previously (29). RNA blot analysis was performed as described in reference 1.

Single-stranded DNA from M13 bacteriophage subclones was sequenced by the dideoxy chain termination method (36), using the Sequenase 2.0 kit (United States Biochemicals Corp., Cleveland, Ohio), with α-^{35}S-dATP (DuPont NEN Research Products) as the label and with commercially available universal primers or other oligonucleotide primers. Both DNA strands in all reported regions were sequenced. Nucleic acid and amino acid sequences were analyzed by using the IBI/Pustell DNA sequence analysis program (International Biotechnologies, Inc., New Haven, Conn.) and the Genetics Computer Group (GCG) program (University of Wisconsin Biotechnology Center, Madison, Wis.). All amino acid sequences shown herein are written in the one-letter IUB amino acid code.

Cloning of the *baiH* gene. Genomic DNA (80 μg) from *Eubacterium* sp. strain VPI 12708 was digested with *Eco*RI (290 U) for 2 h at 37°C and fractionated on a 0.8% agarose gel in a Tris acetate-EDTA buffer system. Fragments in the size range of 1.4 to 2.2 kb were excised from the gel, extracted, ligated into *Eco*RI-digested, dephosphorylated lambda gt11 arms (Bethesda Research Laboratories, Gaithersburg, Md.), and packaged into phage heads by using the Packagene kit (Promega Biotec, Madison, Wis.). The packaged phages were used to infect *E. coli* Y1090r$^-$ (49). Approximately 4,000 plaques were screened by in situ hybridization, using a degenerate oligonucleotide probe specific for the NADH:FOR gene (FOR-2; see Results). DNA (20 μg) from one positive clone was digested with *Eco*RI (80 U) and electrophoresed. The 1.8-kb *Eco*RI fragment, which hybridized to probe FOR-2, was excised, extracted, and ligated into *Eco*RI-digested, dephosphorylated pUC19 DNA (Bethesda Research Laboratories). The ligation mixture was used to transform competent subcloning efficiency cells of *E. coli* DH5α (Bethesda Research Laboratories) in accordance with the instructions of the supplier. *lacZ* colonies were patched onto fresh plates and screened with probe FOR-2, using in situ hybridization. Plasmid DNA from one positive clone, *E. coli* DH5α(pFOR1.8), was isolated. The 0.5-, 0.6-, and 0.7-kb fragments resulting from digestion of the plasmid with *Eco*RI plus *Bam*HI were subcloned into bacteriophages M13mp18 and M13mp19 as described before (1), using *E. coli* JM109 as the host (48).

The downstream portion of the *baiH* gene was synthesized by an inverse polymerase chain reaction (PCR) method. Genomic DNA was digested with *Bam*HI plus *Bcl*I and electrophoresed. Fragments in the size range of 1.2 to 1.6 kb were excised and extracted. A portion of the extracted DNA (100 ng) was self-ligated for 16 h at 15°C. Diverging oligonucleotide primers were designed by using sequence information obtained from the 1.8-kb *Eco*RI fragment above. The forward primer (FOR-4 in Fig. 6) had the sequence 5′ AGTCTGCAGCGCCTGAGTGCCATTGCCAT 3′ and contained an artificial *Pst*I site at the 5′ end. The reverse primer (FOR-5 in Fig. 6) had the sequence 5′ AATGGTACCAG GCCGCGTTCTACGATCT 3′ and contained an artificial *Kpn*I site at the 5′ end. The PCR reaction contained 20 ng of the circularized template, 100 pmol of each primer, and

TABLE 1. Purification of NADH:FOR from *Eubacterium* sp. strain VPI 12708

Purification step[a]	Vol (ml)	Protein (mg)	Activity (U)[b]	Sp act (U/mg)	Purification (fold)	Yield (%)
Cell extract	62.0	2,580	329.2	0.13	1.0	100
DE-52	35.0	1,190	308.9	0.26	2.0	94
Reactive Red 120	37.0	1,060	307.5	0.29	2.2	93
Cibacron Blue A	30.0	72.8	126.0	1.73	13.3	38
DEAE-HPLC (pH 7.0)	19.0	15.2	96.9	6.38	49.1	29
Phenyl-HPLC (pH 7.0)	4.0	0.67	32.4	48.36	372.0	10

[a] Cell extracts were prepared from 16-liter cultures induced by sodium cholate.
[b] One unit of activity is defined as the flavin-dependent oxidation of 1 μmol of NADH per min.

recommended amounts of components provided with the Perkin-Elmer AmpliTaq PCR kit (Perkin-Elmer, Norwalk, Conn.). Temperatures were cycled with a Perkin-Elmer thermal cycler at 94°C (1 min), 55°C (2 min), and 72°C (2 min) for 30 cycles. The 1.4-kb PCR product was agarose gel purified, extracted, digested with *Pst*I plus *Kpn*I, gel purified again, and extracted. The prepared insert was ligated to *Pst*I plus *Kpn*I-digested pUC19, and the ligation mixture was used to transform *E. coli* DH5α competent cells. Colonies were screened for the presence of the 1.4-kb *Pst*I-*Kpn*I insert by in situ hybridization with a 5'-end-labelled oligonucleotide probe (FOR-3 in Fig. 6). Plasmid DNA from one positive clone [*E. coli* DH5α(pFOR1.4)] was isolated, and the 1.4-kb *Pst*I-*Kpn*I insert was purified and subcloned into bacteriophages M13mp18 and M13mp19 as described above.

Nucleotide sequence accession number. The nucleotide and amino acid sequence data presented in this report have been submitted to GenBank (accession number L11069).

RESULTS

Purification of the NADH:FOR. The cholate-inducible NADH:FOR was purified to apparent electrophoretic homogeneity, using a five-step protocol (Table 1). The most effective steps employed were Cibacron Blue A and phenyl-HPLC chromatography, which gave 6- and 7.6-fold purifications and 41 and 34% recoveries, respectively. A purification of 372-fold with a 10% overall recovery was ultimately achieved. Although Reactive Red A agarose chromatography did not result in significant purification of the NADH:FOR, it removed 7α-hydroxysteroid dehydrogenase activity (10), which otherwise contaminated the NADH:FOR preparations. Multiple loadings were necessary to achieve acceptable recoveries from the Cibacron Blue A agarose step, since 20 to 25% of the NADH:FOR activity remained unbound even after 10 passages over the column. The bound NADH:FOR was eluted from this column with 1 M KCl; solutions containing up to 2 M NaCl and 20 mM NAD(H) resulted in enzyme recoveries of <5%.

Three peaks of NADH:FOR activity eluted from the DEAE-HPLC column in the purification protocol (Fig. 1C). The predominant peak eluted at 35 ml, while smaller peaks of activity were detected at 50 and 65 ml; these peaks will be referred to as forms I, II, and III, respectively. To determine the origin of these forms, aliquots of the NADH:FOR preparation from various points in the purification protocol described in Materials and Methods were chromatographed on a DEAE-HPLC column (Fig. 1). Form III was predominant in the pooled DE-52 fractions (Fig. 1A), representing >80% of the total activity. The proportion of form III decreased, and that of form I increased, as the enzyme was purified further. After chromatography of the enzyme on DE-52, Reactive Red A agarose, and Cibacron Blue A agarose (Fig. 1C), form I represented 75 to 80% of the NADH:FOR activity. Form II was only present as a minor peak under these conditions. Changing the pH of the pooled DE-52 fractions from 6.8 to 8.5 (as described in Materials and Methods) did not change the ratio of forms I and III in the DEAE-HPLC column profile.

Forms I and III also displayed different hydrophobic characters on a phenyl-HPLC column (data not shown). When the form I fractions from Fig. 1C were chromatographed on this column, the NADH:FOR activity eluted in a broad peak after the end of a decreasing ammonium sulfate gradient, and most of the activity eluted rapidly when 10%

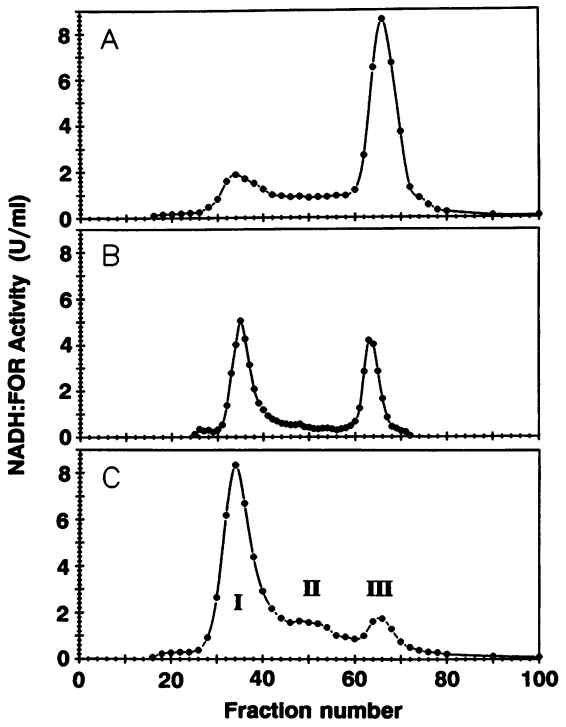

FIG. 1. Effect of purification protocol on the DEAE-HPLC elution profile of NADH:FOR. Protein samples were chromatographed, using a DEAE-HPLC column, at several steps in the purification protocol described in Materials and Methods. Approximately the same amount of NADH:FOR activity was loaded in each panel. Fractions to be loaded were first desalted with Centriprep 10 cartridges, and proteins were eluted as described in Materials and Methods. (A) Proteins from pooled DE-52 fractions; (B) proteins after DE-52 and Reactive Red A chromatography; (C) proteins after DE-52, Reactive Red A, and Cibacron Blue A chromatography. I, II, and III refer to the different forms of the NADH:FOR mentioned in the text.

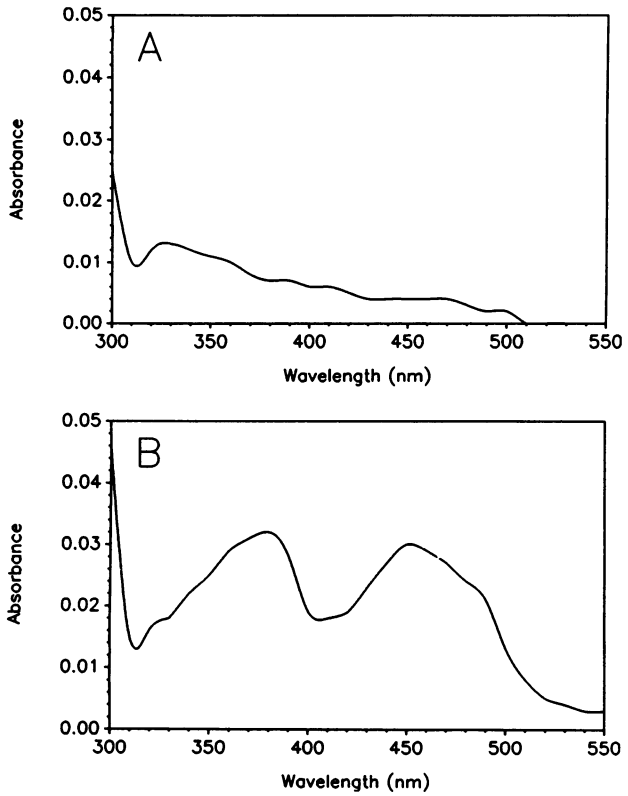

FIG. 2. Absorption spectra of purified NADH:FOR forms I (A) and III (B). The absorption spectra of purified NADH:FOR forms I and III (400 μg each) were determined at 20°C on a Shimadzu UV160U recording spectrophotometer, using a quartz cell with a 1-cm path length.

ethanol was injected onto the column. The form III fractions from Fig. 1C exhibited an elution peak similar to that of form I, but about 50% of the total activity eluted in a new, less hydrophobic peak. The fractions of this new peak were noticeably yellow-brown compared with the colorless fractions containing form I.

The absorption spectra of forms I and III from the phenyl-HPLC column between 300 and 500 nm were compared (Fig. 2). Form III, but not form I, exhibited a typical flavin spectrum, with peaks at 375 and 455 nm. The two forms had approximately equal specific activities in the standard NADH:FOR assay. However, incubation of form I with 150 μM FAD anaerobically (30 min, 25°C) in the presence of NADH, DTT, or 2-mercaptoethanol (all at 1 mM) did not restore the phenyl-HPLC elution profile to that of form III. Inclusion of 5 μM FAD and flavin mononucleotide (FMN) in the DEAE- and phenyl-HPLC column buffers did not change the elution profiles of forms I and III.

Because the amount of form I obtained from DEAE-HPLC was much greater than that of form III (Fig. 1C), form I was used for the purification summarized in Table 1. The enzyme preparation after phenyl-HPLC was judged to be >95% homogeneous by SDS-PAGE and contained a single protein subunit of 72 kDa (Fig. 3). Gel filtration chromatography gave a native molecular mass of 210 kDa (data not shown). These data suggest that the NADH:FOR exists as a trimer of identical subunits. The purified NADH:FOR could not use NADPH in place of NADH as electron donor in the

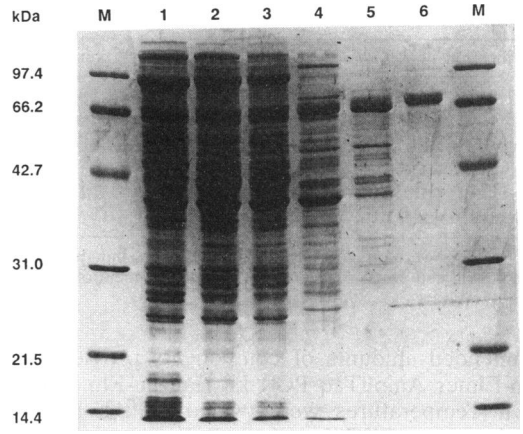

FIG. 3. SDS-PAGE of NADH:FOR. Aliquots from each step of the purification were subjected to electrophoresis, using a 12% acrylamide slab gel. Lanes: M, low-molecular-mass markers (molecular masses indicated at left of gel); 1, cell extract (50 μg); 2, pooled DE-52 fractions (40 μg); 3, pooled Reactive Red A flowthrough fraction (40 μg); 4, pooled Cibacron Blue A fractions (35 μg); 5, pooled DEAE-HPLC (form I) fractions (15 μg); 6, pooled phenyl-HPLC fractions (10 μg).

standard assay. Activity without FAD (i.e., O_2 was the sole electron acceptor) was about 10% of that with FAD. The purified enzyme lost 50% of its original activity when stored at either 4 or −20°C in the presence of 10% (vol/vol) glycerol–1 mM DTT for 2 to 3 weeks.

Effect of various compounds on NADH:FOR activity. The activity of NADH:FOR was very sensitive to the presence of several sulfhydryl inhibitors. Nearly complete inhibition of catalytic activity occurred after incubation of the NADH:FOR with either $CuCl_2$, $HgCl_2$, *N*-ethylmaleimide, or *p*-chloromercuriphenyl sulfonic acid at 1 mM final concentrations (Table 2). The enzyme was also inhibited (approximately 73%) by 1 mM $ZnCl_2$. Similar concentrations of iodoacetate and iodoacetamide, however, failed to substantially inhibit catalytic activity. The metal ion chelators EDTA and

TABLE 2. Effects of various compounds on NADH:FOR activity

Compound	Concn (mM)	% of control[a]
EDTA	5.0	52
EGTA	5.0	101
o-Phenanthroline	2.0	58
NaN_3	10.0	99
$ZnCl_2$	1.0	27
$CuCl_2$	1.0	1
$HgCl_2$	1.0	3
N-Ethylmaleimide	1.0	4
Iodoacetate	1.0	91
Iodoacetamide	1.0	112
p-Chloromercuriphenyl sulfonic acid	0.2	5
Acriflavine	0.2[b]	13
N-Bromosuccinimide	0.2	8

[a] Purified enzyme (10 ng) was preincubated with the indicated amount of the inhibitor or distilled water (control) in standard assay buffer for 10 min at 20°C. Reactions were then initiated by the addition of FAD and NADH and performed as described in Materials and Methods. Enzyme activities are reported as a percentage of the control (2 U/ml) and are the means of triplicate assays.

[b] Because acriflavine is a mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine, its concentration is given in milligrams per milliliter.

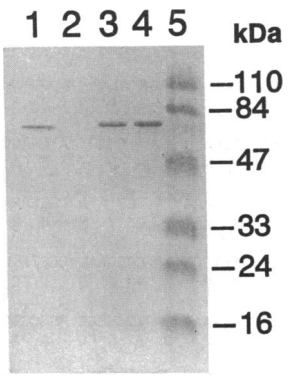

FIG. 4. Western blot analysis of NADH:FOR expression in *Eubacterium* sp. strain VPI 12708. Western blots were performed as described in Materials and Methods. Lanes: 1, cell extract (17 μg) from cholate-induced cells; 2, cell extract (18 μg) from uninduced cells; 3, purified NADH:FOR form I (0.8 μg); 4, purified form III (0.8 μg); 5, prestained molecular mass markers (molecular masses indicated at the right of the blot).

o-phenanthroline, but not [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid (EGTA) or sodium azide, also reduced NADH:FOR activity. In addition, acriflavine (a flavin analog) and *N*-bromosuccinimide were both strongly inhibitory at the concentrations tested.

Expression of NADH:FOR in *Eubacterium* sp. strain VPI 12708. The expression of NADH:FOR in *Eubacterium* sp. strain VPI 12708 was confirmed to be cholate inducible by enzymatic assays and Western blot analysis. Repeated assays of uninduced cell extract revealed no NADH:FOR activity. Western blot analysis revealed that a 72-kDa protein was specifically recognized by the immune serum in cell extracts from cholate-induced cells and in samples of purified NADH:FOR forms I and III (Fig. 4). The specificity of the rabbit antiserum for the NADH:FOR was confirmed by immunoinhibition. The catalytic activity of the purified NADH:FOR was inhibited about 50% after incubation with 150 μl of immune serum. No inhibition of enzymatic activity was detected with preimmune serum.

Mapping the NADH:FOR gene. The sequence of the first 25 amino acids in the N terminus of NADH:FOR was determined by gas phase microsequencing of the purified enzyme. The sequence was MDMKHSR*L*FSPLQIGSLTL XN*F*VXM, where tentatively identified residues are italicized and X represents an undetermined residue. A mixed oligonucleotide (FOR-2), corresponding to the antisense strand encoding the first seven residues of the above, was synthesized and had the following sequence: 5′-TCT(T/A/C/G)GA(G/A)TG(T/C)TTCAT(G/A)TCCAT-3′. Radioactively 5′-end-labelled probe FOR-2 was used in restriction mapping and cloning of the NADH:FOR gene.

The restriction map of the NADH:FOR gene generated with probe FOR-2 (Fig. 5A) matched well that established previously for a large, cholate-inducible operon from *Eubacterium* sp. strain VPI 12708 (29). RNA blots were used to estimate the size of the NADH:FOR mRNA transcript. Probe FOR-2 hybridized to a transcript ≥10 kb in size in RNA samples from cholate-induced but not uninduced cells of *Eubacterium* sp. strain VPI 12708 (Fig. 5B).

Cloning and sequencing of the NADH:FOR gene. The 1.8-kb *Eco*RI fragment which hybridized to probe FOR-2 (Fig. 5A) was cloned into lambda gt11 and then subcloned into plasmid pUC19, generating the recombinant plasmid

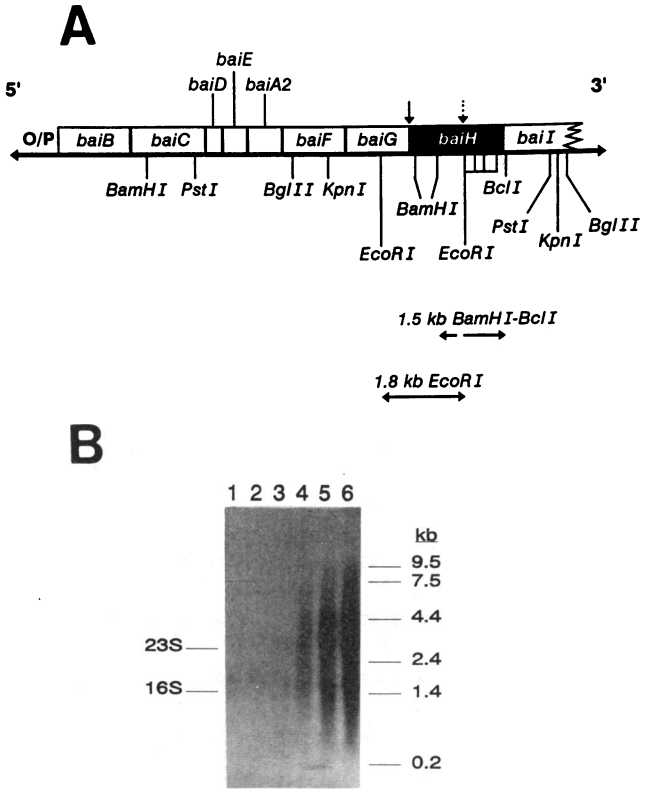

FIG. 5. Location of the NADH:FOR gene (*baiH*) of *Eubacterium* sp. strain VPI 12708 on a large, cholate-inducible operon. (A) Restriction map of the *baiH* gene. Open reading frames are depicted as boxed areas. Open reading frames *baiB*, *-C*, *-D*, *-E*, *-A2*, and *-F* are part of a previously described cholate-inducible operon (29); O/P indicates the putative operator/promoter region for this operon. Open reading frames *baiG*, *baiH*, and *baiI* are inferred from sequence data in Fig. 6. The solid and dotted arrows indicate *baiH*-specific oligonucleotide probes FOR-2 and FOR-3, respectively, used in restriction mapping. Restriction fragments used to clone the *baiH* gene are shown with double-headed arrows. The 1.5-kb *Bam*HI-*Bcl*I fragment was used in an inverse PCR reaction; the gap indicates the region not copied in the PCR reaction (see Results). (B) Northern blot analysis of the *baiH* gene, using probe FOR-2. Lanes: 1, 2, and 3, 10, 20, and 30 μg of total RNA from uninduced cells of *Eubacterium* sp. strain VPI 12708; 4, 5, and 6, 10, 20, and 30 μg of total RNA from cholate-induced cells. RNA size markers are shown at the right. The positions of the 23S and 16S rRNAs are shown at the left.

pFOR1.8. Fragments from *Eco*RI plus *Bam*HI digestion of pFOR1.8 were subcloned into M13 phages for sequencing. Sequence analysis of the 1.8-kb *Eco*RI insert revealed the presence of two truncated open reading frames, one extending from the 5′ *Eco*RI terminus and ending with a stop codon (*baiG*) and the other (*baiH*) beginning with a methionine start codon and continuing through to the 3′ *Eco*RI terminus (Fig. 5A and 6). The deduced amino acid sequence of *baiH* (Fig. 6) matched that obtained by microsequencing of purified NADH:FOR (see above), except that a tentatively identified phenylalanine residue in the latter was replaced by an arginine in the former. This confirmed that *baiH* represented the NADH:FOR gene.

Because the *baiH* gene was truncated at the 3′ end in the 1.8-kb *Eco*RI fragment, the downstream portion was cloned on another fragment. A 1.5-kb *Bam*HI-*Bcl*I fragment over-

FIG. 6. Nucleotide and predicted amino acid sequence of the NADH:FOR gene (*baiH*) and flanking regions. Base pairs 73 to 531 in the DNA sequence have been omitted for brevity. The beginning and end of the *baiH* gene and adjacent open reading frames are indicated below the corresponding amino acid sequences. The predicted amino acid sequence of the *baiH* gene is numbered. Ribosome-binding sites (rbs) and pertinent restriction sites are underlined. Oligonucleotide probes mentioned in the text are depicted as dashed arrows located above the DNA sequence, and the orientation of the probes is indicated by the direction of the arrow. Since probes FOR-3 and FOR-4 overlap, additional bases in FOR-4 are indicated by a double-dashed line.

lapping the 1.8-kb *Eco*RI fragment by 592 bp (Fig. 5A) was identified by DNA blot analysis, using a labelled probe, FOR-3 (Fig. 5A and 6). This fragment was circularized by self-ligation and used as a template for an inverse PCR reaction. Sequence information from the 592-bp overlapping region was used to design diverging primers (FOR-4 and -5 in Fig. 6) with artificial *Pst*I and *Kpn*I restriction sites. The use of these primers and the circularized template in the PCR reaction generated a 1.4-kb *Pst*I-*Kpn*I insert, which was subcloned into pUC19 and then into M13 phages for sequencing.

Sequence analysis (Fig. 6) revealed that the *baiH* gene was 1,983 bp in length and encoded a polypeptide of 661 amino acid residues with a molecular weight of 72,006. This agreed with the subunit relative molecular weight of purified NADH:FOR as determined by SDS-PAGE. A potential open reading frame, *baiI*, was found downstream from the *baiH* gene (Fig. 5A and 6).

Global amino acid sequence homologies. Computer data base searches indicated that the predicted amino acid sequence of the *baiH* gene shared global homology with those of four other proteins: NADH oxidase from *Thermoanaerobium brockii* (26); trimethylamine dehydrogenase from the facultative methylotroph bacterium W3A1 (4); Old Yellow

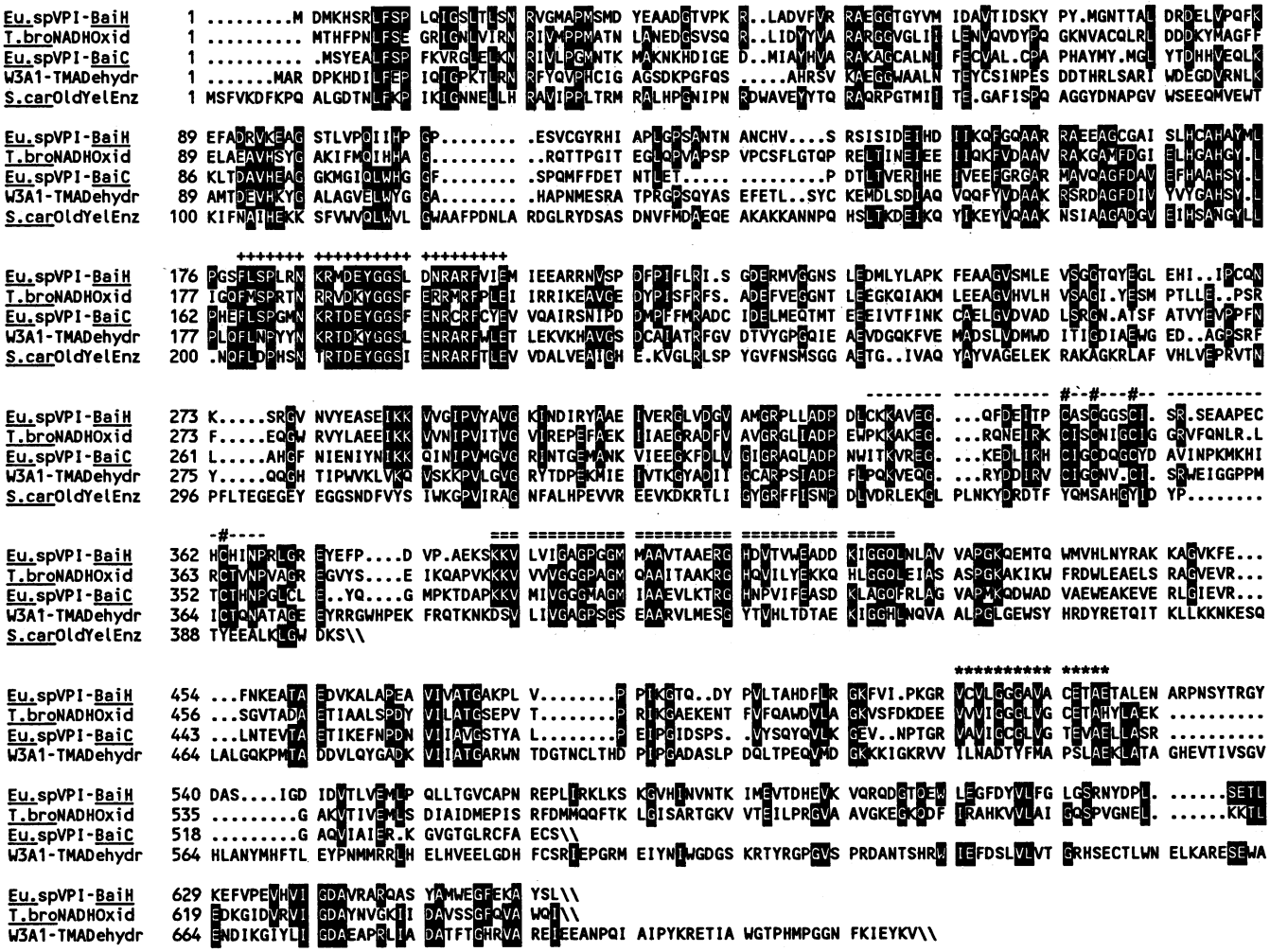

FIG. 7. Alignment of the predicted amino acid sequence of the *baiH* gene of *Eubacterium* sp. strain VPI 12708 with other predicted amino acid sequences. The GenBank data base was screened with the GCG TFASTA (33) program to identify homologous deduced amino acid sequences, which were then aligned with the GCG PILEUP program (gap weight = 3.0; gap length weight = 0.1). Periods indicate gaps introduced by the program for optimal alignment. Residues are numbered at the left of each line. Residues conserved in at least two of three of the sequences are outlined in black. The sequences compared are listed below, along with the abbreviations used; GenBank accession numbers are indicated in brackets. *Eu.* spVPI-*BaiH*, NADH:FOR encoded by the *baiH* gene of *Eubacterium* sp. strain VPI 12708 (this paper) [L11069]; *T.bro*NADHOxid, NADH oxidase from *T. brockii* (26) [X67220]; *Eu.* spVPI-*BaiC*, the polypeptide encoded by the *baiC* gene of *Eubacterium* sp. strain VPI 12708 (29) [M36292]; W3A1-TMADehydr, trimethylamine dehydrogenase from the methylotrophic bacterium W3A1 (4) [S111873]; *S.car*OldYelEnz, Old Yellow Enzyme from *S. carlsbergensis* (35) [S64034]. Symbols: \\, carboxy terminus of the amino acid sequence; +, highly conserved region; –, cysteine-rich region (conserved cysteines are marked with #); =, putative FAD binding domain; *, segment with homology to a domain in *E. coli* thioredoxin reductase (34).

Enzyme from *Saccharomyces carlsbergensis* (35); and the predicted polypeptide product of the *baiC* gene (29), which is located upstream from the *baiH* gene in the large, cholate-inducible operon of *Eubacterium* sp. strain VPI 12708 (Fig. 5A). An alignment of these sequences is shown in Fig. 7. All of the sequence matches showed greater than 24% identity and 48% similarity when aligned with the GCG GAP program and had Z scores of greater than 10 (considered to indicate significant homology) when analyzed with the RDF2 program (33). The best match (optimized Z score of 102) was with NADH oxidase, which shared 38.3% identity and 57.5% similarity with the *baiH* predicted amino acid sequence.

Features of the amino acid sequence. The amino acid segment FlsPxxNkRtDeYGGSxenRaRFxlE (perfectly conserved residues are capitalized and x represents any residue) was highly conserved among all five sequences compared in Fig. 7. Computer data base searches with the GCG FASTA (33), WORDSEARCH, PROFILESEARCH, or MOTIFS programs failed to identify any other sequences with significant homology to this segment.

A cluster of four cysteine residues was conserved in all of the sequences except that of Old Yellow Enzyme (Fig. 7). The spacing of these cysteines was as follows: C(2x)C(2–3x)C(11–13x)C. A portion of the *baiH* predicted amino acid sequence surrounding this cluster (residues 329 to 367) contained six cysteine residues, including the four mentioned above. Searches with the MOTIFS program (two

TABLE 3. Biochemical characteristics of dehydrogenases homologous to NADH:FOR from *Eubacterium* sp. strain VPI 12708[a]

Enzyme, organism	Mol wt (10^3)[b]		α_x[c]	Cofactors per subunit (mol/mol)[d]		Electron donor	Electron acceptor(s)[e]	Reference(s)
	Subunit	Native						
NADH:FOR, *Eubacterium* sp. strain VPI 12708	72.0	210	α3	xFe/xS?	xFlavin	NADH	Dyes, menadione, ferricyanide, O_2, FAD, FMN, riboflavin	25; this paper
NADH oxidase, *T. brockii*	71.3	≥450	α6	2Fe/xS	2 FAD	NADH	Dyes, menadione, ferricyanide, O_2	26, 27
Trimethylamine dehydrogenase, methylotrophic bacterium W3A1	81.6	163	α2	4Fe/4S	1 FMN, 1 ADP	$(CH_3)_3N$	Electron transfer flavoprotein	2, 4, 38, 39
Old Yellow Enzyme, *S. carlsbergensis*	45.0	90	α2	ND	1 FMN	NADPH	Dyes, menadione, ferricyanide, O_2, duroquinone, benzoquinone, coenzyme Q_1	31, 35
baiC gene product, *Eubacterium* sp. strain VPI 12708	59.5	?	?	?	?	?	?	29

[a] ?, no data available.
[b] Subunit molecular weights were calculated from deduced amino acid sequences. Native molecular weights are experimentally determined; that of *S. carlsbergensis* Old Yellow Enzyme is inferred from its dimeric structure (35).
[c] αx refers to the subunit composition of the enzymes.
[d] Fe/S, iron and acid-labile sulfide atoms; x indicates that the number of either per subunit has not been determined. ND, none detected.
[e] Dyes include tetrazolium salts, dichlorophenol-indophenol, and methylene blue.

mismatches allowed) indicated that this region was similar to the 4Fe/4S binding sequence present in ferredoxins and other iron-sulfur proteins [C(2x)C(2x)C(3x)CP]. Searches with the FASTA program indicated homology of this region (≥40% identity over 20 residues) with a cysteine cluster in several mammalian metallothioneins, particularly in the spacing of the cysteines: C(13–14x)C(2x)C(3x)C(8–9x)CxC.

A well-conserved domain (corresponding to residues 384 to 420 of the *baiH* predicted amino acid sequence) was present in all of the sequences in Fig. 7, except that of Old Yellow Enzyme, which terminated just upstream from this area. When analyzed with the FASTA program, this domain in the sequence from *baiH* showed considerable homology (up to 68% identity over 25 residues) with the FAD dinucleotide-binding domain of disulfide reductases, including lipoamide dehydrogenases, mercuric reductases, and glutathione reductases (40).

The subsequence VCVLGGGAVACETA from the *baiH* predicted amino acid sequence (residues 510 to 523, Fig. 7) matched a segment in thioredoxin reductase from *E. coli*, VAVIGGGNTAVEEA (34), in 8 of 14 residues, with one conservative substitution. This segment in thioredoxin reductase is proposed to represent an NADPH binding site (34) by analogy with that in human glutathione reductase (22). Similar subsequences were present in the sequences from *baiC* and NADH oxidase, but not in that of trimethylamine dehydrogenase.

DISCUSSION

Cell extracts of *Eubacterium* sp. strain VPI 12708 have a cholate-inducible NADH:FOR, which has been partially purified and characterized previously (25). In the present study, the purified NADH:FOR was confirmed to be NADH dependent, and Western blot analysis of cell extracts showed that expression of the 72-kDa NADH:FOR polypeptide is also cholate inducible. The effects of various sulfhydryl reagents and metal chelators on NADH:FOR activity are also similar to those reported previously (25). The partially purified enzyme is inhibited 25% by 1 mM *p*-chloromercuribenzoate (a sulfhydryl-reactive compound) and over 50% by 5 mM *o*-phenanthroline (a nonheme iron chelator). The purified enzyme was even more sensitive to the presence of sulfhydryl-reactive compounds, with greater than 90% inhibition seen with 1 mM $CuCl_2$, $HgCl_2$, or *N*-ethylmaleimide. The inhibition by sulfhydryl-reactive compounds suggests that one or more cysteine residues may be catalytically important for this enzyme. The inhibition by metal ion chelators such as *o*-phenanthroline and EDTA suggests that this enzyme is a metalloprotein. Given the affinity of *o*-phenanthroline for nonheme iron, the NADH:FOR may contain iron-sulfur (Fe/S) centers. The inhibition of enzyme activity by EDTA may account for some of the difficulty experienced previously in purifying this enzyme, since EDTA was included in all purification buffers at a concentration of 0.1 mM (25).

Activity of the purified NADH:FOR was strongly inhibited by the flavin analog acriflavine. Form III, but not form I, of the purified enzyme exhibited a typical flavin absorption spectrum. These data suggest that form III contains a bound flavin, which readily dissociates during purification, generating form I. Attempts to reconstitute form I with FAD to regenerate form III under reduced conditions were unsuccessful, and inclusion of FAD and FMN in the purification buffers did not prevent the production of form I. However, the endogenous flavin was apparently not essential for NADH:FOR activity, because the specific activities of forms I and III in the standard assay were approximately equal.

The restriction map of the NADH:FOR gene (*baiH*) closely resembled that of a cholate-inducible, polycistronic operon described previously in *Eubacterium* sp. strain VPI 12708 (29). This operon contains at least six genes (*baiB* through *baiF* in Fig. 5A), but the 3′ end has not yet been located (29). Sequence analysis of the 667 bp upstream from the *baiH* gene (Fig. 6) revealed no potential promoter sequences but suggested the presence of another open reading frame, *baiG*. Sequencing work in progress suggests that this open reading frame extends from the *baiF* to the *baiH* gene with no intervening promoter sequence (unpublished data). RNA blot analysis with a *baiH*-specific probe indicated that the transcript for this gene is cholate inducible and ≥10 kb long. These data strongly suggest that the *baiH* gene is located on the previously described cholate-inducible operon (29).

NADH:FOR showed significant amino acid sequence homology with three other oxidoreductases: NADH oxidase from *T. brockii* (26, 27); trimethylamine dehydrogenase from the facultatively methylotrophic bacterium W3A1 (4); and Old Yellow Enzyme from *S. carlsbergensis* (35) (Fig. 7). Some biochemical characteristics of these enzymes are compared in Table 3. They vary widely in subunit molecular weight, but those of NADH:FOR and NADH oxidase are nearly identical. All but Old Yellow Enzyme 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 serve as electron donors for three of the enzymes: NADH:FOR, NADH oxidase, and Old Yellow Enzyme (also known as an NADPH dehydrogenase). The enzymes reduce a variety of nonphysiological electron acceptors. However, the physiological electron acceptor for trimethylamine dehydrogenase is a FAD-containing electron transfer flavoprotein with a native molecular weight of 77,000 (38) which may donate electrons to ubiquinone in the electron transport chain, as does its eucaryotic counterpart (11). Old Yellow Enzyme from brewer's bottom yeast reduces certain nonphysiological quinones, including menadione, benzoquinone, duroquinone, and coenzyme Q_1, but the in vivo function of this enzyme is unknown (31).

Several features were conserved among the amino acid sequences of the above enzymes (Fig. 7). The conserved cluster of four cysteines probably represents ligands for an Fe/S center. In fact, X-ray crystallography studies have shown that these four cysteines in trimethylamine dehydrogenase are ligands for its 4Fe/4S center (2). Old Yellow Enzyme, which does not contain Fe/S centers, lacks this conserved cysteine cluster. The spacing of the cysteines in the conserved cluster, C(2x)C(2–3x)C(11–13x)C, is different from the signature sequence for 4Fe/4S centers of ferredoxins, C(2x)C(2x)C(3x)CP, which may reflect differences in the folding and intramolecular electron transfer in these proteins. The sequence surrounding the four-cysteine cluster in NADH:FOR includes two additional cysteine residues. The spacing of these six cysteines is almost identical to that of mammalian metallothioneins, which function to bind heavy metals (21). A putative FAD (dinucleotide)-binding domain (40) was present in NADH:FOR, NADH oxidase, trimethylamine dehydrogenase, and the *baiC* gene product. The sequence of Old Yellow Enzyme, which contains FMN but not FAD, ends just upstream from this domain. Trimethylamine dehydrogenase also contains FMN rather than FAD. However, it also has one molecule of ADP per subunit, which binds within this domain (24). The amino acid segment VxVxGxGxVxxExA was present in NADH:FOR, NADH oxidase, and the *baiC* gene product, but not in trimethylamine dehydrogenase or Old Yellow Enzyme. This is similar to a domain in *E. coli* thioredoxin reductase which is thought to represent an NADPH-binding site (34), by analogy to that of human glutathione reductase (22).

Bile acid 7α-dehydroxylation in *Eubacterium* sp. strain VPI 12708 proceeds via a multistep, cholate-inducible pathway (3, 8, 20). The large, cholate-inducible operon which appears to include the NADH:FOR (*baiH*) gene contains at least six other genes thought to be involved in 7α-dehydroxylation. For example, the *baiB* gene encodes a bile acid-coenzyme A ligase (28), and the *baiA2* gene encodes a putative 3α-hydroxysteroid dehydrogenase (47). NADH:FOR may provide reducing equivalents for the reductive steps of bile acid 7α-dehydroxylation, which are stimulated by the addition of reduced flavins (45). The *baiC* gene located on this operon (Fig. 5A) encodes a 59.5-kDa polypeptide homologous to the NADH:FOR (29), suggesting that these two gene products are functionally related. The cloning and sequencing of the NADH:FOR gene will facilitate further studies of the physiological function and biochemical structure of this enzyme.

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