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Ethylbenzene Dehydrogenase, a Novel Hydrocarbon-oxidizing Molybdenum/Iron-Sulfur/Heme Enzyme*

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The initial enzyme of ethylbenzene metabolism in denitrifying *Azoarcus* strain EbN1, ethylbenzene dehydrogenase, was purified and characterized. The soluble periplasmic enzyme is the first known enzyme oxidizing a nonactivated hydrocarbon without molecular oxygen as cosubstrate. It is a novel molybdenum/iron-sulfur/heme protein of 155 kDa, which consists of three subunits (96, 43, and 23 kDa) in an $\alpha\beta\gamma$ structure. The N-terminal amino acid sequence of the α subunit is similar to that of other molybdenum proteins such as selenate reductase from the related species *Thauera selenatis*. Ethylbenzene dehydrogenase is unique in that it oxidizes the hydrocarbon ethylbenzene, a compound without functional groups, to (*S*)-1-phenylethanol. Formation of the product was evident by coupling to an enantiomer-specific (*S*)-1-phenylethanol dehydrogenase from the same organism. The apparent K_m of the enzyme for ethylbenzene is very low at <2 μM. Oxygen does not affect ethylbenzene dehydrogenase activity in extracts but inactivates the purified enzyme, if the heme b cofactor is in the reduced state. A variant of ethylbenzene dehydrogenase exhibiting significant activity also with the homolog *n*-propylbenzene was detected in a related *Azoarcus* strain (PbN1).

Three bacterial species capable of anaerobic degradation of the aromatic hydrocarbon ethylbenzene are known to date. All of these are denitrifying bacteria that belong to the genus *Azoarcus* of the β-proteobacteria. For one of these strains, *Azoarcus* sp. EB-1, ethylbenzene is the only known hydrocarbon utilized as growth substrate (1). The other two strains utilize either ethylbenzene or an alternative hydrocarbon compound, namely toluene (strain EbN1) or *n*-propylbenzene (strain PbN1) (2). The proposed pathway of anaerobic degradation of ethylbenzene by these bacteria is shown in Fig. 1. It is initiated by a novel biochemical reaction, namely an oxygen-independent oxidation of ethylbenzene to (*S*)-1-phenylethanol. This intermediate is then oxidized further to acetophenone by an alcohol dehydrogenase (1–4). Activities of an ethylbenzene-oxidizing enzyme and an enantio-specific (*S*)-1-phenylethanol dehydrogenase have been reported in cell extracts of strain EB-1 (4), and a substrate-specific (*S*)-1-phenylethanol dehydrogenase has been purified and characterized from strain EbN1.[1] The intermediate acetophenone is apparently degraded further by carboxylation to benzoylacetate to yield benzoyl-CoA and acetyl-CoA eventually (Fig. 1; for review, see Ref. 5). The catabolic pathway of *n*-propylbenzene in strain PbN1 is supposed to be analogous to that of ethylbenzene, yielding benzoyl-CoA and propionyl-CoA as intermediates (2). Toluene degradation in strain EbN1 proceeds via a completely different pathway and involves the formation of benzylsuccinate from toluene and fumarate as initial reaction (3, 6, 7).

In this report, we analyze the biochemical properties of the first enzyme of anaerobic ethylbenzene metabolism, ethylbenzene dehydrogenase. The enzyme was purified and shown to be a new periplasmic molybdenum/iron-sulfur/heme protein that oxidizes ethylbenzene stereospecifically to (*S*)-1-phenylethanol. We also provide evidence that the same enzyme catalyzes anaerobic oxidation of ethylbenzene and *n*-propylbenzene.

EXPERIMENTAL PROCEDURES

Growth of Bacteria and Preparation of Cell Extracts—Strain EbN1 was isolated previously from an enrichment culture on ethylbenzene by Rabus and Widdel (2). Growth of the bacteria in 1–2-liter scale cultures was performed as described previously (2, 3). Cells were grown by subsequent transfer for at least 30 generations on the same substrate prior to harvesting for the described experiments. Harvesting was performed anoxically while the cultures were in the exponential growth phase. Fermenter cultures (200 liters) were set up as described previously (3) and run in fed-batch mode with a growth-limiting and exponentially increasing feeding rate of nitrate and discontinuous supply of ethylbenzene. Growth rates of 0.015–0.025 h^{-1} and cell yields of 200–300 g (wet mass)/fermenter were usually obtained. Extract preparation was usually performed aerobically. Cells (10 g, wet mass) were suspended in 10 ml of water and passed through a French pressure cell at 137 megapascals. Cell debris and membranes were removed by ultracentrifugation (1 h at 100,000 × *g*). Washed membrane fractions were prepared from the supernatant of a 20,000 × *g* centrifugation step, which was centrifuged at 100,000 × *g* for 1 h. The pellet was washed and resuspended in the same volume of basal buffer (10 mM Tris-Cl, 1 mM $MgCl_2$, 10% glycerol, pH 7.5). For anaerobic extract preparation, all solutions were degassed and stored under nitrogen, and all handling steps were performed in an anaerobic glove box as described earlier (3). Strain PbN1 (2) was grown in 2-liter bottles under the same conditions as described for strain EbN1. The hydrocarbon substrates were added to the cultures in an inert carrier phase (2,2,4,4,6,8,8-heptamethylnonane) containing 2% (v/v) ethylbenzene or 4% (v/v) *n*-propylbenzene. Shortest doubling times of 10.5 h on ethylbenzene and 12 h on *n*-propylbenzene were recorded.

Enzyme Assays—Ethylbenzene dehydrogenase was routinely assayed in 100 mM Tris-Cl buffer (pH 7.5) containing 0.2 mM ferricenium hexafluorophosphate as electron acceptor. Enzyme solution was added, and the reactions were started by adding ethylbenzene or *n*-propylbenzene (final concentration, 100 μM) from saturated aqueous solutions, which contained 2 mM ethylbenzene (8) or 1 mM *n*-propylbenzene (9). Decrease of absorption of the ferricenium ion was followed at 290 nm ($\Delta\epsilon$ = 9,000 M^{-1} cm^{-1}). The tests were routinely performed under aerobic conditions because identical activities were observed in control tests under anaerobic conditions. To assess the pH optimum of ethylbenzene dehydrogenase, the enzyme assay was also performed in sodium phosphate buffers within a pH range of 6.0–8.0. Alternative assays for ethylbenzene oxidation were set up with 0.1 mM dichlorophe-

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[1] O. Kniemeyer and J. Heider, submitted for publication.

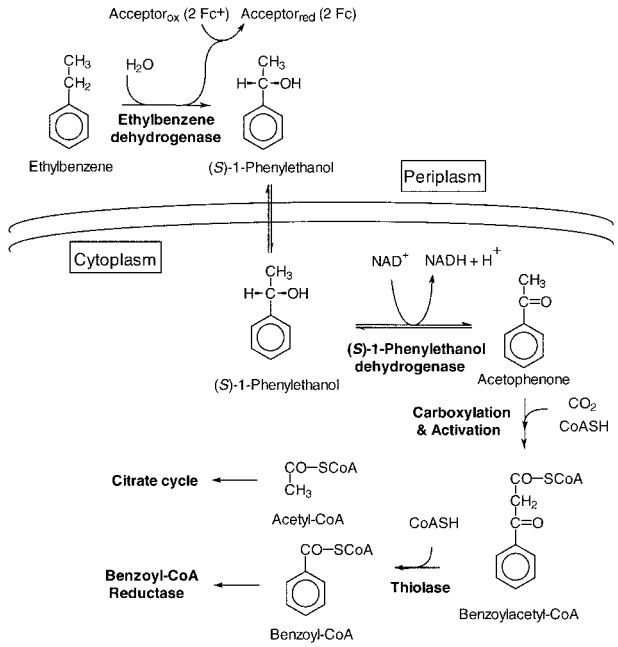

FIG. 1. **Proposed anaerobic degradation pathway of ethylbenzene in strain EbN1.** Fc^+, ferricenium; Fc, ferrocene.

nol indophenol as electron acceptor in the presence and absence of the redox mediator phenazine methosulfate (0.05 mM). These tests were performed under anaerobic conditions as described above and were monitored for dichlorophenol indophenol reduction at 546 nm. Reversibility of the ethylbenzene dehydrogenase reaction was tested under strictly anaerobic conditions in 100 mM Tris-Cl buffer (pH 7.5), containing 1 mM methyl viologen and 0.5 mM dithionite. Oxidation of reduced methyl viologen was followed at 710 nm (ϵ = 2,400 M^{-1} cm^{-1}). After adding the enzyme, the reaction was started by adding 1 mM (*S*)-1-phenylethanol. The same buffer was also used to assess the purified enzyme for possible selenate reductase or nitrate reductase activities. In these cases, the reaction was started by the addition of 1 mM respective electron acceptor. (*S*)-1-Phenylethanol dehydrogenase activity was assayed in 100 mM Tris-Cl buffer (pH 7.5) containing 2 mM $MgCl_2$, 0.5 mM NAD, and 1 mM (*S*)-1-phenylethanol and enzyme. Malate dehydrogenase activity was measured in 100 mM potassium phosphate buffer (100 mM, pH 7) containing 0.25 mM NADH, 0.2 mM oxaloacetate, and cell extract. Reduction of NAD^+ or oxidation of NADH was followed photometrically at 365 nm (ϵ = 3.4 mM^{-1} cm^{-1}).

Enzyme Purification—All column chromatography steps were performed in an anaerobic glove box with an FPLC System (Amersham Pharmacia Biotech). Extract of ethylbenzene-grown cells of strain EbN1 (20 ml of a 100,000 × *g* supernatant) was applied to a DEAE-Sepharose column (Amersham Pharmacia Biotech; 2.2-cm diameter, 50-ml volume), which had been equilibrated with basal buffer (2 mM Tris acetate buffer, pH 8.0, and 10% w/v glycerol). The column was washed at a flow rate of 5 ml min^{-1} for 2 column volumes and eluted with a gradient from 0 to 50 mM KCl in basal buffer over 500 ml. Fractions of 7 ml were collected. Ethylbenzene dehydrogenase activity eluted in a volume of 80 ml between 40 and 50 mM KCl. A yield of 77% and an enrichment factor of 20 were obtained after this step (see Table III). The active fractions were applied on a ceramic hydroxyapatite column (10 ml; Bio-Rad, Hercules, CA), which had been equilibrated with basal buffer. The column was washed with 2 volumes of basal buffer. A gradient over 100 ml was then applied from 0 to 300 mM potassium phosphate, and fractions of 5 ml were collected. Enzyme activity eluted in a volume of 40 ml when 160–250 mM potassium phosphate was applied. Active fractions were pooled, and ethylbenzene dehydrogenase was concentrated by ammonium sulfate precipitation under anaerobic conditions (60% saturation of ammonium sulfate).

Separation of Subcellular Compartments—Cells of strain EbN1 were grown and harvested as described above. Spheroplasts were formed by using a modification of previous procedures (10, 11). Freshly harvested cells (0.8 g, wet mass) were resuspended in 64 ml of TS buffer (30 mM Tris-Cl, 30% sucrose, pH 8). EDTA (9 mM final concentration) and lysozyme (2.6×10^6 units) were added, and the suspension was incubated on ice for 120 min to produce spheroplasts. Periplasmic proteins were prepared by centrifugation of the spheroplast suspension for 30 min at 16,000 × *g*. Most of the periplasmic proteins were recovered in the supernatant, whereas the pellet contained the intact spheroplasts. These were washed in TS buffer, suspended in 25 ml of buffer (20 mM Tris-Cl, 10 mM $MgCl_2$, 10% glycerol, pH 8) containing 10 mg DNase I and lysed by one passage through a French pressure cell. The membrane and soluble cytoplasmic fractions of the cell lysate were separated by centrifugation at 100,000 × *g*.

Other Methods—Protein concentrations were determined according to Lowry (12) or by the Coomassie dye binding test (12) with bovine serum albumin as standard, and discontinuous SDS-PAGE[2] was performed in 15% (w/v) polyacrylamide gels according to standard procedures (12). Molecular mass standards were phosphorylase *b*, bovine serum albumin, ovalbumin, lactate dehydrogenase, carbonic anhydrase, trypsin inhibitor, and lysozyme. Gels were analyzed by the ImageMaster® one-dimensional software (Amersham Pharmacia Biotech). UV-visible spectra were recorded with a λ2 photometer (PerkinElmer Life Sciences). Cytochrome *c* content in subcellular fractions was analyzed and calculated as described (13). The native molecular mass of ethylbenzene dehydrogenase was determined by gel filtration on a calibrated Superdex 200 column (Amersham Pharmacia Biotech) and by analysis of purified enzyme on native polyacrylamide gels. Gels containing five different polyacrylamide concentrations between 6 and 8% (w/v) were used, and ovalbumin and the monomer, dimer, trimer, and tetramer bands of bovine serum albumin were used as standards for a Ferguson plot (12). Photometric quantitation of molybdenum, tungsten (14), iron (15), and inorganic sulfide (16) was performed by standard chemical techniques. Additionally, a simultaneous determination of 32 elements in purified enzyme was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Jarrel Ash Plasma Comp 750 instrument at the center of Complex Carbohydrate Research, University of Georgia. For protein microsequencing, cell extract or purified enzyme was separated by SDS-PAGE and blotted on a polyvinylidene difluoride membrane (Pro Blott, Applied Biosystems, Weiterstadt, Germany) using a Semi-Phor (model TE77) semidry blotting device as described in (17). Proteins on polyvinylidene difluoride membrane were stained by Coomassie Blue R-250. The proteins were subjected to Edman degradation microsequencing (Procise 492 Sequencer, Applied Biosystems) with repetitive yields of >96%. Ferricenium hexafluorophosphate was synthesized following a published procedure (18); all other chemicals were from Fluka, Sigma (Deisenhofen, Germany), or Merck (Darmstadt, Germany) and were of the highest available purity.

RESULTS

Ethylbenzene and n-Propylbenzene Dehydrogenase Activities in Strain EbN1—A photometric enzyme assay was developed for the first enzyme of anaerobic ethylbenzene degradation of strain EbN1, ethylbenzene dehydrogenase. The artificial electron acceptors dichlorophenol indophenol or phenazine methosulfate were tested without success for coupling to ethylbenzene oxidation. However, significant activity of an ethylbenzene dehydrogenase was detected in extracts of ethylbenzene-grown cells with the ferricenium cation as electron acceptor. The assay was dependent on the amount of protein, and a pH optimum of 7.0 was determined. Identical activities were obtained under oxic and strictly anoxic conditions, indicating that molecular oxygen is not required for ethylbenzene oxidation. No decrease in activity was recorded when extracts were adjusted to pH 5.5 or to pH 9.0 prior to starting the enzyme assay. Ethylbenzene dehydrogenase activity was detected exclusively in the soluble fraction after 100,000 × *g* centrifugation; no activity was found in washed membrane fractions. With untreated cell extracts, an ethylbenzene:ferricenium stoichiometry (ethylbenzene:electron ratio) of 1:3.9 was determined, indicating that ethylbenzene is oxidized to acetophenone in these assays. Assuming a stoichiometry of 1:4, the specific ethylbenzene oxidation rate in 100,000 × *g* supernatants was 22 ± 4 nmol min^{-1} (mg of protein)$^{-1}$. This value is

[2] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; ICP-OES, inductively coupled plasma optical emission spectroscopy.

TABLE I

Ethylbenzene dehydrogenase and n-propylbenzene dehydrogenase activities in 100,000 × g extracts of differently grown cells of Azoarcus sp. strains EbN1 and PbN1

No activity was measured in washed membrane fractions. The values are means of at least two measurements. Standard deviations were <11%. ND, not determined.

Strain	Growth substrate	Specific activity with	
		Ethylbenzene	*n*-Propylbenzene
		nmol/min/mg protein	
EbN1	Ethylbenzene	22.1	3.5
EbN1	(*S*)-1-Phenylethanol	1.5	ND
EbN1	(*R*)-1-Phenylethanol	1.1	ND
EbN1	(*R*/*S*)-1-Phenylethanol	3.0	ND
EbN1	Acetophenone	1.4	ND
EbN1	Benzoate	<0.1	ND
PbN1	*n*-Propylbenzene	45.5	30.0
PbN1	Ethylbenzene	52.5	32.5
PbN1	Benzoate	<0.1	<0.1

close to a calculated minimum substrate degradation rate of 28 nmol min^{-1} (mg of protein)$^{-1}$ required in growing cells at the time of harvesting (doubling time 11 h). Very low ethylbenzene dehydrogenase activity was recorded in extracts of cells grown on (*S*)-, (*R*)-, or racemic 1-phenylethanol, or on acetophenone, and none in benzoate-grown cells (Table I). Clear activity was also observed when ethylbenzene was replaced by *n*-propylbenzene in the assays with ethylbenzene-grown cells, albeit only at about 15% of the activity measured with ethylbenzene (Table I).

Ethylbenzene and n-Propylbenzene Dehydrogenase Activities in Strain PbN1—Cells of strain PbN1, which were grown in 2-liter cultures on ethylbenzene or *n*-propylbenzene, were also tested for dehydrogenase activities for ethylbenzene and *n*-propylbenzene. Ethylbenzene-grown cells of strain PbN1 contained two times higher specific activities of ethylbenzene dehydrogenase than observed in strain EbN1 (Table I). In extracts of strain PbN1, *n*-propylbenzene was oxidized at a rate of 62–66% of that measured with ethylbenzene. If strain PbN1 was grown on *n*-propylbenzene, the specific activity of the enzyme was slightly lower, but the ratio of the rates with *n*-propylbenzene or ethylbenzene as substrates was nearly the same as with ethylbenzene-grown cells (Table I). These findings suggest that ethylbenzene and *n*-propylbenzene are oxidized by a common enzyme. Specific activities for both substrates were sufficient to explain the observed growth rates of strain PbN1 on either hydrocarbon.

Protein Patterns of Cells of Strain EbN1 and PbN1 Grown on Different Substrates—Ethylbenzene-grown cells of strain EbN1 contained large amounts of several substrate-induced polypeptides, which were lacking in cells grown on 1-phenylethanol, the next intermediate of the metabolic pathway. The most prominent ethylbenzene-induced polypeptides visible in cell extracts separated by SDS-PAGE are shown in Fig. 2. Several other substrate-induced polypeptides were also observed in cells grown on 1-phenylethanol or acetophenone compared with benzoate-grown cells (Fig. 2), indicating sequential induction of the catabolic enzymes of anaerobic ethylbenzene metabolism. Extracts of ethylbenzene- or *n*-propylbenzene-grown cells of strain PbN1 showed no obvious difference in polypeptide patterns. Several prominent substrate-induced polypeptides of identical sizes were observed in cells grown on either hydrocarbon substrate compared with benzoate-grown cells (Fig. 2).

Stoichiometry and Stereospecificity of Ethylbenzene Dehydrogenase—Proteins in extracts of ethylbenzene-grown cells of *Azoarcus* sp. strain EbN1 were separated initially by chromatography on DEAE-Sepharose in 10 mM Tris-Cl buffer (pH 7.5). Under these conditions, 100% of ethylbenzene dehydrogenase activity was found in the flow-through fractions. (*R*)- and (*S*)-

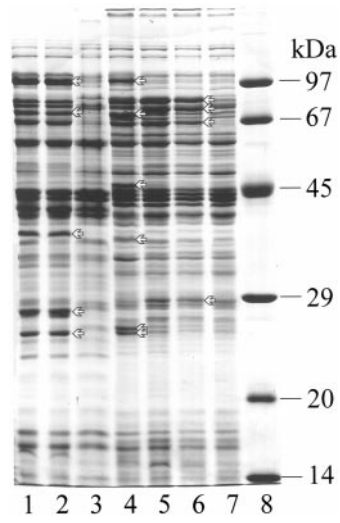

FIG. 2. **Protein patterns of strains EbN1 and PbN1 cells grown on different substrates.** Coomassie Blue-stained SDS-PAGE (12% gel, w/v), in which cell extracts (40 μg of protein/lane) were separated, is shown. Substrate-induced polypeptides are indicated by *arrows*. Apparent masses of the alkylbenzene-induced polypeptides were 96, 71, 44, 33, 24, and 23 kDa in strain EbN1 and 96, 73, 34, 24, and 23 kDa in strain PbN1. Substrate-induced proteins in 1-phenylethanol- and acetophenone-grown cells of strain EbN1 had apparent masses of 81, 76, 66, and 29 kDa (see Ref. 24). *Lanes 1–3*, strain PbN1 grown on ethylbenzene (*lane 1*), *n*-propylbenzene (*lane 2*), and benzoate (*lane 3*). *Lanes 4–7*, strain EbN1 grown on ethylbenzene (*lane 4*), (*R*, *S*)-1-phenylethanol (*lane 5*), acetophenone (*lane 6*), and benzoate (*lane 7*). Sizes of the marker proteins in *lane 8* are indicated.

1-Phenylethanol dehydrogenases from the cell extract were retained on the column,[1] thus allowing complete separation of the first two enzymes of ethylbenzene degradation. Using the separated enzymes, we determined whether the four-electron oxidation of ethylbenzene to acetophenone, as detected in cell extracts, is catalyzed by ethylbenzene dehydrogenase alone, or whether it depends on the subsequent (*S*)-1-phenylethanol dehydrogenase reaction. Enzyme assays with ethylbenzene dehydrogenase containing flow-through fractions, which are devoid of (*S*)-1-phenylethanol dehydrogenase, showed an ethylbenzene:ferricenium stoichiometry of 1:2.3. The stoichiometry changed to 1:4.1 when the tests were supplied with NAD^+ and purified (*S*)-1-phenylethanol dehydrogenase[1] and remained at 1:2.3 when the same experiment was performed in the absence of NAD^+. Therefore, ethylbenzene dehydrogenase catalyzes a two-electron oxidation of ethylbenzene, and stereospecifically produces (*S*)-1-phenylethanol, which is subsequently oxidized to acetophenone by (*S*)-1-phenylethanol dehydrogenase in cell extract. The NADH generated by the alcohol dehydrogenase is apparently reoxidized by an NADH:acceptor oxidoreductase with ferricenium as electron acceptor.

Subcellular Localization of Ethylbenzene Dehydrogenase—After generating spheroplasts from freshly harvested cells of ethylbenzene-grown strain EbN1 by lysozyme/EDTA treatment in isotonic Tris-Cl/sucrose buffer, 94% of the ethylbenzene dehydrogenase was released into the medium (Table II). The periplasmic marker protein cytochrome *c* was also largely released in these experiments, as determined from difference spectra (dithionite-reduced minus oxidized), whereas 93% of the cytoplasmic marker enzyme malate dehydrogenase was retained in the spheroplasts (Table II). Determination of (*S*)-1-phenylethanol dehydrogenase activities in the subcellular fractions showed that 20% of this enzyme was released, and about 80% was retained in the spheroplasts (Table II). This indicated that ethylbenzene oxidation to (*S*)-1-phenylethanol occurs in the periplasm, whereas further oxidation of (*S*)-1-phenyletha-

TABLE II
Subcellular distribution of ethylbenzene dehydrogenase in Azoarcus strain EbN1

Cells were grown anaerobically in the presence of nitrate and ethylbenzene. Specific activities are given in units of 1 nmol substrate/min/mg protein. Mean values of at least two measurements are given. Standard deviations were <14%.

Fraction	Ethylbenzene dehydrogenase	Malate dehydrogenase	(*S*)-1-Phenylethanol-DH	Cytochrome *c*
	nmol substrate/min mg protein			*nmol/mg protein*
Periplasmic	75	2,400	19	1.9
Cytoplasmic	1.8	30,000	70	0.05

nol to acetophenone occurs in the cytoplasm. This is corroborated by the stoichiometries of ethylbenzene oxidation *versus* ferricenium reduction in the different subcellular fractions. In cytoplasmic fractions, an ethylbenzene:ferricenium stoichiometry of 1:3.2 was determined, indicating coupling of the residual ethylbenzene dehydrogenase activity with (*S*)-1-phenylethanol dehydrogenase, whereas the periplasmic fractions showed an ethylbenzene:ferricenium stoichiometry of 1:1.9.

Purification of Ethylbenzene Dehydrogenase from Strain EbN1—Because ethylbenzene dehydrogenase did not bind to DEAE-Sepharose columns when 10 mM Tris-Cl buffer (pH 7.5) was used, the elution buffer was changed to a chloride-free 2 mM Tris acetate buffer (pH 7.5). Under these conditions, the enzyme bound to the DEAE-column and was then eluted from the column by a linear KCl gradient. Although enzyme activity in extracts was not affected by air, enriched enzyme from the first column quickly lost activity under oxic conditions. Therefore, purification of the enzyme was performed under anoxic conditions, which resulted in typical yields of >75% and an enrichment factor of 20 after the first column (Table II). Chromatography on ceramic hydroxyapatite (Bio-Rad) was performed as second purification step. The pooled enzyme eluted from the DEAE-Sepharose column was applied directly on this column and eluted by a linear potassium phosphate gradient (pH 7.5). A typical enrichment factor of 24 at a yield of 20% was obtained after this step. The enzyme was essentially pure after the second column, as shown by SDS-PAGE. A summary of the purification scheme is given in Table III.

Molecular Properties of Ethylbenzene Dehydrogenase—Ethylbenzene dehydrogenase consists of three subunits of 96, 43, and 23 kDa, as revealed by SDS-PAGE of the purified enzyme (Fig. 3). The apparent native molecular mass of the enzyme was determined as 155 ± 15 kDa by gel filtration and Ferguson plot analysis of native polyacrylamide gels. These values are consistent with an assumed $\alpha\beta\gamma$ composition of the enzyme. The N-terminal amino acid sequences of the three subunits were determined from enzyme that had been separated by SDS-PAGE. The N terminus of the α subunit of purified ethylbenzene dehydrogenase was blocked, whereas short sequences of the β and γ subunits were obtained. However, a sequence of the α subunit was obtained when the ethylbenzene-induced 96-kDa polypeptide was cut out from blotted cell extracts and used for sequencing. The N-terminal amino acid sequence of the α subunit (GTKAPGYASWEDIYRKEWKWDKVN) was highly similar to that of other molybdoproteins such as selenate reductase of *Thauera selenatis* (63% identity) or several nitrate reductases (*e.g.* 63% identity with nitrate reductase subunit 1 of *Haloarcula marismortui*). The sequences obtained for the other subunits (β, *X*GP*XX*YLRP; and γ, *X*KAKRVPGGKELLLDL) did not show significant matches with known proteins.

Additionally, molar contents of molybdenum, iron, and acid-labile sulfide were determined in purified ethylbenzene dehydrogenase. Molybdenum content in ethylbenzene dehydrogenase was determined as 0.9 ± 0.1 mol (mol of enzyme)$^{-1}$ by colorimetric assay, and as 1.2 ± 0.1 mol (mol of enzyme)$^{-1}$ by ICP-OES. Iron was determined chemically at a stoichiometry of 17 ± 2 mol (mol of enzyme)$^{-1}$ compared with 16 ± 1 mol of iron (mol of enzyme)$^{-1}$ determined by ICP-OES. Finally, colorimetric analysis of acid-labile sulfide yielded 12 ± 4 mol S^{2-} (mol of enzyme)$^{-1}$. These values are consistent with the presence of one molybdenum, four [Fe_4S_4] clusters, and one heme in ethylbenzene dehydrogenase, as reported previously for selenate reductase of *T. selenatis* (19, 20). Other metals detected by ICP-OES in significant amounts were magnesium and calcium (2.5 mol/mol each), but no further transition metals or selenium were present in purified enzyme.

Spectral Properties of Ethylbenzene Dehydrogenase—UV-visible spectroscopic analysis of purified ethylbenzene dehydrogenase showed a complex spectrum. The spectrum of the purified enzyme showed a shoulder around 400 nm and distinct absorption maxima at 424, 528, and 559 nm, which indicated the presence of a reduced heme b cofactor. After anaerobic oxidation of the enzyme by stoichiometric concentrations of ferricenium hexafluorophosphate, the α and β peaks of the tentatively identified heme at 559 and 528 nm disappeared, and the Soret band at 424 nm was shifted to 414 nm (Fig. 4). The difference spectrum of reduced and oxidized enzyme was indeed indicative of the presence of a heme b cofactor (Fig. 4). Addition of stoichiometric concentrations of ethylbenzene to ferricenium-oxidized enzyme resulted in restoration of the spectrum of reduced enzyme (Fig. 4). The spectra of the substrate-reduced enzyme and the enzyme obtained from the final column were identical, suggesting that ethylbenzene dehydrogenase was purified in the completely reduced form. Treatment of the reduced enzyme with 0.2 mM dithionite did not result in further reduction of the heme cofactor but resulted in further bleaching of the absorption between 400 and 500 nm (Fig. 4). This is indicative of the presence of iron-sulfur clusters in ethylbenzene dehydrogenase. These clusters are obviously not completely reduced by the substrate and need a strong chemical reductant such as dithionite for being entirely reduced. The heme content of the enzyme was determined as 0.95 mol/mol from dithionite-reduced enzyme, using an ϵ of 34.7 mM^{-1} cm^{-1} for the α band (21).

Catalytic Properties of Ethylbenzene Dehydrogenase—Purified ethylbenzene dehydrogenase catalyzed the ferricenium-dependent oxidation of ethylbenzene to (*S*)-1-phenylethanol. The specific activity recorded for purified enzyme corresponds to a very low catalytic number of 1.2 s^{-1}, a value similar to that recorded for benzoyl-CoA reductase and phenylacetyl-CoA:acceptor oxidoreductase from *Thauera aromatica* (22, 23). Ethylbenzene dehydrogenase activity was already saturated at extremely low ethylbenzene concentrations, which prevented the accurate determination of the K_m value for ethylbenzene. An approximation of <2 μM for the K_m value was derived from the residual substrate concentration under half-saturating turnover conditions (for estimation, see Fig. 5). About 15% of the activity recorded with ethylbenzene was obtained when *n*-propylbenzene was used as substrate. The ratio of activities with the two substrates did not change significantly between cell extract and purified enzyme. Other aromatic substrates, such

TABLE III
Purification of ethylbenzene dehydrogenase

A typical purification scheme is shown.

	Volume	Total activity	Total protein	Specific activity	Yield	Enrichment
	ml	*μmol/min*	*mg*	*nmol/min/mg*	*%*	*fold*
Extract	12	7,700	480	16	100	1
DEAE-Sepharose	80	5,920	19	312	77	20
Hydroxyapatite	40	1,560	4	390	20	24

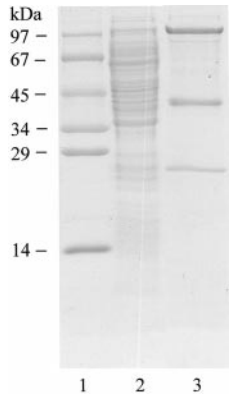

FIG. 3. **SDS-PAGE of active pools during purification of ethylbenzene dehydrogenase.** *Lane 1*, marker proteins (sizes given in *left margin*); *lane 2*, crude extract (50 μg of protein); *lane 3*, purified enzyme (10 μg) after chromatography on hydroxyapatite.

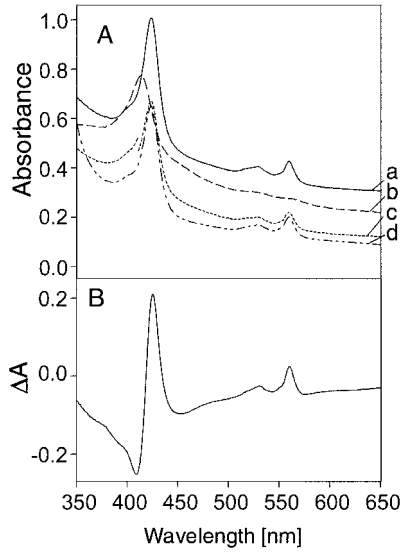

FIG. 4. **UV-visible spectrum of ethylbenzene dehydrogenase.** *Panel A*, spectrum of purified enzyme (protein concentration 0.94 mg ml^{-1}) directly after isolation under anoxic conditions (*a*), after anaerobic oxidation with 15 μM ferricenium hexafluorophosphate (*b*), after re-reduction of ferricenium-oxidized enzyme with 10 μM ethylbenzene (*c*), and after vigorous reduction with 0.2 mM dithionite (*d*). For better visibility, *spectra b–d* were offset along the *y* axis. *Panel B*, difference spectrum of reduced enzyme (*a* in *panel A*) and ferricenium-oxidized enzyme (*b* in *panel A*).

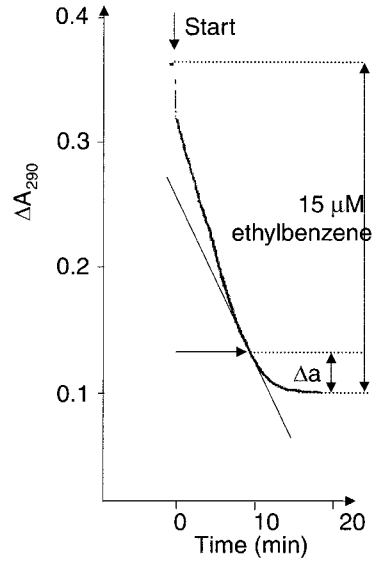

FIG. 5. **Half-maximal activity of ethylbenzene dehydrogenase.** A photometric assay was performed recording the reduction of ferricenium at 290 nm (the initial ferricenium concentration was 200 μM). The reaction was started by the addition of 15 μM ethylbenzene. The *horizontal arrow* marks the time point when the rate of absorbance decrease was half-maximal. The corresponding residual concentration of ethylbenzene was calculated from the absorbance difference of ferricenium (Δ*A*) between this time point and the end of the reaction.

as toluene, *p*-cymene, phenylacetate, (*R*,*S*)-1-phenylethanol or 2-phenylethanol, were not oxidized by ethylbenzene dehydrogenase. The potential of ethylbenzene dehydrogenase to catalyze the reversed reaction was tested by an anaerobic enzyme assay with reduced methyl viologen as electron donor and (*S*)-1-phenylethanol as starting substrate. No reduction of (*S*)-1-phenylethanol to ethylbenzene was detected by this test, suggesting that the reaction of ethylbenzene dehydrogenase is irreversible under physiological conditions. Ethylbenzene dehydrogenase also did not catalyze methyl viologen-dependent reduction of selenate or nitrate, despite the strong similarity of the N-terminal sequences of the respective α subunits.

Inhibition of Ethylbenzene Dehydrogenase—Ethylbenzene dehydrogenase was not inhibited in assays containing sodium azide or sodium cyanide (1 mM each). Addition of cyanide to the assay buffer caused a strong nonenzymatic background reaction, probably by sequestering iron from the ferricenium into a cyanide complex, but enzyme activities were still measurable reliably after starting with ethylbenzene. Whereas ethylbenzene dehydrogenase activity in cell extracts was not affected by aerobic extract preparation and incubation in air for up to 12 h, purified enzyme, which was apparently in the reduced state (see above), was inactivated irreversibly by incubation in air with a half-life time of 7 min. This inactivation was prevented efficiently by addition of the artificial electron acceptor ferricenium hexafluorophosphate (1 mM) to the enzyme preparations. Under these conditions, >90% of the enzyme activity was still present after 2 h and >70% after a 6-h incubation in air. Because ferricenium has been shown to convert the heme cofactor of ethylbenzene dehydrogenase to the oxidized form, it can be concluded that the reduced heme is most likely responsible for inactivation by oxygen. The enzyme is probably held oxidized in cell extracts by natural electron acceptors and acquires oxygen sensitivity when these electron acceptors are removed during purification.

DISCUSSION

The pathway of anaerobic ethylbenzene metabolism is initiated by two consecutive two-electron oxidation steps of ethylbenzene to (*S*)-1-phenylethanol and further to acetophenone (1–3). In this study, we analyzed the first enzyme of the predicted pathway, ethylbenzene dehydrogenase. To our knowledge, ethylbenzene dehydrogenase is the first described enzyme that catalyzes oxygen-independent hydroxylation of a hydrocarbon, namely, of an apolar compound without functional groups. Enantiomer specificity of ethylbenzene dehydrogenase was demonstrated by coupling the reaction to that of purified (*S*)-1-phenylethanol dehydrogenase. This matches the product reported previously for a different *Azoarcus* strain (4). Ethylbenzene dehydrogenase was induced specifically in cells grown anaerobically on ethylbenzene, and only very low activities were measured in cells grown on 1-phenylethanol or acetophenone. Three of the ethylbenzene-induced polypeptides described in this study showed molecular masses identical to those of the subunits of ethylbenzene dehydrogenase (96, 44, and 23 kDa).

Cells of the ethylbenzene-metabolizing strain EbN1 contained an enzyme exhibiting *n*-propylbenzene dehydrogenase activity at 15% of the specific activity measured with ethylbenzene. In contrast, cells of the ethylbenzene and *n*-propylbenzene-metabolizing strain PbN1 contained an enzyme exhibiting high activity with either ethylbenzene or *n*-propylbenzene. Similar ratios of activity with the two hydrocarbons in cells grown on either substrate strongly suggest that the same enzyme is used for metabolism of ethylbenzene and *n*-propylbenzene. This assumption is supported by the apparent identity of the polypeptide patterns of ethylbenzene an *n*-propylbenzene grown cells. Ethylbenzene dehydrogenase showed no activity with toluene as substrate, which is consistent with previous observations that strain EbN1 catabolizes toluene via a completely different pathway, namely, the addition of the methyl group to fumarate (5–7).

The present data suggest that ethylbenzene oxidation occurs in the periplasm, whereas the product, (*S*)-1-phenylethanol, is oxidized further in the cytoplasm, as evident from the use of NAD^+ as electron acceptor. It is unknown presently whether and how 1-phenylethanol is transported into the cytoplasm. A passive diffusion of 1-phenylethanol as a hydrophobic compound via the cytoplasmic membrane appears principally possible. The advantage of a periplasmic location of ethylbenzene dehydrogenase for the organism is unknown. One may speculate that scavenging of the poorly water-soluble ethylbenzene is more effective outside of the cytoplasmic membrane. The low K_m value (<2 μM) of ethylbenzene dehydrogenase supports the assumption that the capacity of the enzyme for effective substrate binding is an important factor in the metabolism of this hydrocarbon.

Even though the redox potential of the 1-phenylethanol/ethylbenzene couple is around +0.03 V relative to standard hydrogen electrode (estimated from thermodynamic data of other hypothetical alcohol/hydrocarbon couples), ethylbenzene oxidation was only observed in this study with an electron acceptor of significantly higher redox potential (ferricenium/ferrocene, $E^0 = +0.38$ V). One may assume that redox centers in the enzyme as well as the natural electron acceptor have high redox potentials to achieve reasonable oxidation rates with the relatively inert hydrocarbon substrate. Redox centers of high midpoint potential in the enzyme may offer a possible explanation for the observed irreversibility of ethylbenzene dehydrogenation, even in tests with strongly reducing electron donors (*e.g.* methyl viologen$^+$/methyl viologen, $E^0 = -0.446$ V). Because ethylbenzene dehydrogenase is a periplasmic enzyme, a possible natural acceptor in the *Azoarcus* strains could be cytochrome *c*. The properties of ethylbenzene dehydrogenase from strain EbN1 are in contrast to the recently reported benzoquinone dependence of ethylbenzene oxidation in strain EB-1. Reaction rates in this strain were 3-fold lower than those reported here, and the enzyme was membrane-associated and not induced in ethylbenzene-grown cells (4). Apparently, there are different types of ethylbenzene dehydrogenases in different strains of denitrifiers.

Ethylbenzene dehydrogenase is a new molybdenum/iron-sulfur/heme enzyme, which is composed of three subunits. In analogy to other known three-subunit molybdoenzymes, it may be assumed that the α subunit contains the molybdenum cofactor; the β subunit carries the Fe/S clusters; and the γ subunit, the heme cofactor. The enzyme, which is most similar to ethylbenzene dehydrogenase with respect to subunit composition, cofactor content and location, is presumably the recently characterized selenate reductase from a closely related species, *T. selenatis* (19, 20). However, ethylbenzene dehydrogenase did not catalyze reduction of selenate or nitrate. Another recently characterized molybdoprotein, phenylacetyl-CoA:acceptor oxidoreductase from *T. aromatica*, catalyzes a similar dehydrogenation reaction with a polar aromatic substrate, but this enzyme is membrane-bound, devoid of a heme cofactor, and apparently catalyzes the four-electron oxidation of phenylacetyl-CoA to phenylglyoxylate, with coupling to quinones and without release of intermediates (23).

A striking finding is the discrepancy between the stability of ethylbenzene dehydrogenase under air in cell extracts and the fast inactivation of the purified enzyme by oxygen. We showed that the enzyme becomes relatively insensitive to air by anaerobic oxidation of the heme b cofactor, suggesting that the fully reduced heme in the enzyme may generate damaging oxygen metabolites. In cell extract, ethylbenzene dehydrogenase is probably kept oxidized by interaction with its natural electron acceptor and only becomes reduced when the electron acceptor is removed during purification.

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Ethylbenzene Dehydrogenase, a Novel Hydrocarbon-oxidizing Molybdenum/Iron-Sulfur/Heme Enzyme

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