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Purification and Characterization of NADH Oxidase from *Serpulina (Treponema) hyodysenteriae*

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NADH oxidase (EC 1.6.99.3) was purified from cell lysates of *Serpulina (Treponema) hyodysenteriae* B204 by differential ultracentrifugation, ammonium sulfate precipitation, and chromatography on anion-exchange, dye-ligand-affinity, and size-exclusion columns. Purified NADH oxidase had a specific activity 119-fold higher than that of cell lysates and migrated as a single band during denaturing gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]). The enzyme was a monomeric protein with an estimated molecular mass of 47 to 48 kDa, as determined by SDS-PAGE and size-exclusion chromatography. Optimum enzyme activity occurred in buffers with a pH between 5.5 and 7.0. In the presence of oxygen, β-NADH but not α-NADH, α-NADPH, or β-NADPH was rapidly oxidized by the enzyme (K_m = 10 μM β-NADH; V_{max} = 110 μmol β-NADH min^{-1} mg of $protein^{-1}$). Oxygen was the only identified electron acceptor for the enzyme. On isoelectric focusing gels, the enzyme separated into three subforms, with isoelectric pH values of 5.25, 5.35, and 5.45. Purified NADH oxidase had a typical flavoprotein absorption spectrum, with peak absorbances at wavelengths of 274, 376, and 448 nm. Flavin adenine dinucleotide was identified as a cofactor and was noncovalently associated with the enzyme at a molar ratio of 1:1. Assays of the enzyme after various chemical treatments indicated that a flavin cofactor and a sulfhydryl group(s), but not a metal cofactor, were essential for activity. Hydrogen peroxide and superoxide were not yielded in significant amounts by the *S. hyodysenteriae* NADH oxidase, indirect evidence that the enzyme produces water from reduction of oxygen with NADH. The N-terminal amino acid sequence of the NADH oxidase was determined to be MKVIVIGCNHAGTWAAK. In its biochemical properties, the NADH oxidase of *S. hyodysenteriae* resembles the NADH oxidase of another intestinal bacterium, *Enterococcus faecalis*.

Serpulina (Treponema) hyodysenteriae is the etiologic agent of swine dysentery. Cells of this anaerobic spirochete colonize, invade, and damage the mucosal epithelium of the swine cecum and colon (7, 14, 17, 43). The cecal and colonic mucosa consists of oxygen-respiring cells.

Although *S. hyodysenteriae* is considered an anaerobe, *S. hyodysenteriae* cells growing beneath a 1% oxygen atmosphere consume substrate amounts (1.6 to 2.0 μmol/ml of culture) of oxygen (40). Oxygen consumption by cell suspensions is accompanied by decreased yields of H_2 and butyrate, products of NADH oxidation (38). *S. hyodysenteriae* cell lysates contain NADH oxidase, NADH peroxidase, and superoxide dismutase activities (38). On the basis of its high specific activity, NADH oxidase is a major, and perhaps the only, mechanism for the consumption of oxygen by *S. hyodysenteriae*.

NADH oxidases (EC 1.6.99.3) have been found in diverse bacteria, both aerobes and anaerobes. These enzymes mediate the direct transfer of electrons from NADH to molecular oxygen without intermediate electron carriers (34). Characterized NADH oxidases are flavoproteins and are either soluble or membrane associated. NADH oxidases are a means for bacteria to contend with or take advantage of oxygen in their environment (1, 5, 10, 11, 18, 23, 33).

There is little understanding of the adaptations contributing to the success of *S. hyodysenteriae* as a mucosal pathogen of swine, although lipopolysaccharide (22), endotoxin (9), hemolysins (19, 44), and motility (14) have been considered to play a role. Our current hypothesis is that oxygen metabolism and an ability to withstand external oxidative stress are essential characteristics for *S. hyodysenteriae* to colonize respiring tissues of the swine cecum and colon. In view of this hypothesis, we have investigated an NADH oxidase from *S. hyodysenteriae* B204 cells. The *S. hyodysenteriae* NADH oxidase is a single-subunit, flavin adenine dinucleotide (FAD)-linked enzyme with a molecular mass of 47 to 48 kDa. The purification and additional characteristics of this enzyme are reported in this article.

MATERIALS AND METHODS

Culture conditions. Cells of *S. hyodysenteriae* B204 were routinely cultured in BHIS broth as described previously (38, 39). For enzyme purification, bacteria were cultured in 12 liters of BHIS broth in a bench-top fermentor (Bio-Kulture fermentor assembly; Fermentation Design, Inc.) with a 14-liter-capacity fermentor vessel (New Brunswick model 19). Calf serum (final concentration, 5% [vol/vol]) was used in place of fetal calf serum. Sterile medium was kept beneath a flow of filtered oxygen-free nitrogen (1 liter/min) delivered through the headspace of the fermentor. The broth was inoculated with *S. hyodysenteriae* cells (250 ml) in the exponential phase of growth (1×10^8 to 3×10^8 cells per ml, by direct microscope counts). After 1 h, the headspace gas was changed to sterile compressed air (0.2 liter/min). Cultures were stirred (350 rpm) with the magnetic stirring assembly of the fermentor. Approximately 20 h after inoculation, cells in the exponential growth phase were harvested from the culture broth (culture A_{620}, 1.3 to 1.5; 18-mm-pathlength cuvette). Cells were harvested by centrifugation (4°C, 5,000 × *g*, 5 min), resuspended, and washed once with

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500 ml of cold (5°C) sodium phosphate buffer (50 mM, pH 7.0). The final cell pellet (22 to 27 g [wet weight]) was stored frozen (−20°C) for 1 to 10 weeks before cell lysis.

Preparation and ultracentrifugation of cell lysates. Cell pellets from at least two fermentor harvests were processed at the same time. Partially thawed pellets were added to cold (5°C) PBCF buffer (1 ml of buffer per g of cell pellet). PBCF buffer was prepared fresh daily by mixing sodium phosphate buffer (0.2 M, pH 7.0) containing 0.3 mM FAD with an equal volume of L-cysteine–HCl solution (0.125 M, pH 7.0). The cell-buffer mixture was gently stirred in an ice bath on a magnetic stirring platform until the cells were completely resuspended (30 min). During stirring, DNase (2,000 Kunitz units in 1 ml of 0.15 M NaCl) and phenylmethylsulfonyl fluoride (4 μg in 100 μl of acetone) were added. Suspended cells were lysed in a chilled (5°C) French pressure cell (Aminco Instrument Co., Silver Spring, Md.) at 7,500 lb/in^2. Microscopic examination indicated lysis of >99.9% of the cells. The cell lysate was placed in a room-temperature water bath for 15 min to hasten DNA digestion. Cell lysates were then centrifuged (2 h, 147,000 × *g*, 5°C) to remove unbroken cells, cell membranes, and medium debris. After ultracentrifugation of cell lysates, NADH oxidase activity remained in the supernatant or S_1 fraction (38).

Ammonium sulfate fractionation. After ultracentrifugation of cell lysates, the S_1 fraction was diluted 10-fold with cold PBCF buffer to give a solution containing 2.0 to 2.5 mg of protein per ml. Proteins were precipitated from this solution with $(NH_4)_2SO_4$ in the saturation ranges of 0 to 40, 40 to 60, 60 to 80, and 80 to 90% (35) and pelleted by centrifugation (5°C, 10,000 × *g*, 10 min). The precipitated protein from the 60 to 80% fraction contained most (80 to 90%) of the oxidase activity and was stored in the centrifuge bottle at 5°C overnight.

Ion-exchange high-performance liquid chromatography. Precipitated protein in the 60 to 80% salt-precipitated fraction was dissolved in 4 ml of PBCF buffer. A PD-10 size-exclusion column (Pharmacia-LKB Biotechnology, Inc., Piscataway, N.J.) was used to remove $(NH_4)_2SO_4$ and replace the phosphate buffer with 10 mM Tris buffer (pH 8.0) prepared from Trizma-HCl and Trizma base. A 1.5-ml volume of dissolved protein gave 3.5 ml of column eluate. Combined eluates of two or three desalted samples were concentrated at 5°C to about 1 ml in a Centriprep-30 ultrafiltration concentrator (Amicon Co., Danvers, Mass.), and the concentrated sample was injected onto a preparative ion-exchange column (TSK-DEAE-5-PW, 150 by 21.5 mm; Bio-Rad Laboratories, Richmond, Calif.). NADH oxidase was eluted at 22°C from the column by increasing concentrations of KCl in 10 mM Tris buffer (see Fig. 1A). Active fractions (10 ml) were combined, mixed with an equal volume of double-strength PBCF buffer, and placed on ice. This solution was concentrated at least 20-fold with a Centriprep-30 unit. Glycerol was added (final concentration, 25% [vol/vol]), and the solution was stored at −80°C.

Dye-ligand affinity chromatography. After ion-exchange chromatography, the oxidase preparation was passed through a PD-10 column, and a 3.5-ml eluate was applied to a Blue A affinity column equilibrated with 10 mM sodium phosphate buffer (pH 7.0, 5°C). The column was a hand-packed, dye-ligand agarose column (50 by 16 mm, Matrex Gel Blue A; Amicon). A step gradient of KCl in 10 mM phosphate buffer was used to elute oxidase activity (see Fig. 1B). Eight active fractions (2 ml) were pooled and concentrated 30-fold with a Centriprep-30 unit. Although the oxidase activity could be purified by using a hand-packed dye-ligand agarose column, attempts at dye-ligand chromatography with high-performance liquid chromatography (HPLC) columns (Supelco Progel-TSK Blue-5PW and Pierce Selectispher-10-Cibacron Blue F3G-A) were unsuccessful.

Size-exclusion high-performance liquid chromatography. Concentrated fractions (0.3 to 0.5 ml) from the dye-ligand affinity column were chromatographed at 22°C on a preparative HPLC size-exclusion column (21.5 by 300 mm, Spherogel-TSK G3000SW; Beckman Instruments, Inc., Fullerton, Calif.) fitted with a precolumn (21.5 by 7.5 mm). The migration of protein standards (gel filtration calibration kit for low-molecular-weight proteins; Pharmacia-LKB) was used to calibrate the column and estimate the molecular mass of NADH oxidase. The elution buffer was 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 2 ml/min. Oxidase activity eluted in three 1-ml fractions at about 30 to 32 min after injection. Active fractions were concentrated approximately 10-fold (Centricon-30), mixed with glycerol (final concentration, 25% [vol/vol]), and stored frozen (−80°C).

NADH oxidase assay. Throughout this article, NADH is used interchangeably with β-NADH. The rate of NADH oxidation (decrease in A_{340}) was recorded with a Gilford Response II spectrophotometer with an automatic cuvette positioner (Ciba-Corning Diagnostics Corp., Medfield, Mass.). All assays were carried out at room temperature (22°C). The assay buffer contained FAD (final concentration, 10 μM) and NADH (final concentration, 0.2 mM) in 50 mM sodium phosphate buffer (pH 7.0). An NADH stock solution (50×; β-NADH; Sigma catalog no. N-8129) was prepared in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 8.5). FAD was eliminated from assays used to characterize pure enzyme. The assay buffer supplied sufficient oxygen to measure enzyme activity. The amount of NADH oxidized was estimated by using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ at 340 nm for NADH. Most assays were begun by adding an oxidase sample (1 to 10 μl containing 60 to 150 ng of NADH oxidase protein) to 3 ml of assay buffer in a spectrophotometer cuvette (1-cm pathlength). The linear decrease in absorbance was measured for 4 to 6 min. To easily compare oxidase-specific activities at different purification stages and in experiments with different assay conditions, enzyme preparations were diluted to give a "standard" decrease in A_{340} of 0.02 U/min.

Protein assay. Protein concentrations were determined by the Lowry technique after precipitation of protein with trichloroacetic acid (26). This precipitation eliminated interfering substances (L-cysteine) and concentrated small amounts of protein. Bovine serum albumin (BSA; Sigma catalog no. A-4378) was used as a standard. A BSA standard solution of 0.5 mg of BSA per ml of water in a 1-cm-pathlength cuvette had an A_{280} of 0.33 ± 0.01.

Isoelectric focusing. The isoelectric pH of NADH oxidase was estimated with an isoelectric focusing gel (Phast-System; Pharmacia-LKB). Precast gels (PhastGel IEF, pH 4 to 6.5) and the isoelectric pH calibration kit (Low pI Kit, pH 2.5 to 6.5) were purchased from Pharmacia-LKB. The conditions for electrophoresis and staining of proteins were recommended by the manufacturer.

An activity assay to detect NADH oxidase in gels was based on that described by Seymour and Lazarus (37). Immediately after isoelectric focusing, the gel was placed in 20 ml of ice-cold 0.1 M sodium phosphate buffer (pH 7.0). After 5 min, the gel was submerged in 20 ml of the same buffer at room temperature. A freshly made solution of

NADH (0.5 ml; 50 μg of NADH per ml of 0.1 M phosphate buffer [pH 7.0]) was mixed into the gel buffer. At 1-min intervals, the gel was removed and held over a UV light box (Fotodyne Inc., New Berlin, Wis.) to detect bands of NADH oxidase activity. The NADH diffusing into the gel fluoresced under UV light. Dark bands appeared in the gel where the enzyme oxidized NADH to NAD. When bands were clearly visible, the gel was dipped into distilled water (5°C), placed on the UV light box, and photographed. The gel was then stained for protein.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor purification of NADH oxidase and estimate the molecular mass of the enzyme. Electrophoresis was carried out at room temperature at 175 V for 1 h with a 3.75% acrylamide stacking gel over a 10% acrylamide resolving gel (0.75-mm thick). A vertical gel electrophoresis apparatus (Mini-Protean II), power supply (model 250/2.5), molecular mass standards (low range), and all electrophoresis reagents were purchased from Bio-Rad. Methods for casting gels, electrophoresis, preparing buffers, and staining proteins with Coomassie blue were as described in the manufacturer's recommendations.

H_2O_2 and superoxide assays. Hydrogen peroxide, a potential product of NADH oxidation, was assayed as follows (6). NADH oxidase assay mixes containing 350 ng of oxidase and 500 nmol of NADH in 2 ml of sodium phosphate buffer (50 mM, pH 7.0) were incubated at 22°C until NADH was completely oxidized (approximately 10 min; $A_{340} = 0.01$). A 1-ml solution containing 2.5 mM 4-aminoantipyrine, 0.17 M phenol, and horseradish peroxidase (0.4 U; Sigma P-8250) was then added, and the resulting solution was incubated at 22°C until the peroxidase reaction was complete (maximum A_{510}). The final A_{510} of the mixture was compared with that of reaction mixes lacking oxidase but containing known amounts of hydrogen peroxide. In control assays, hydrogen peroxide was added (2.5, 5, or 50 nmol per assay mix) at the beginning of the NADH oxidase reaction to ensure that peroxide was stable in the presence of NADH oxidase.

Superoxide production in NADH oxidase assays was determined by monitoring cytochrome *c* reduction (by the increase in A_{550} at room temperature) (13). Assay mixes (3 ml) contained 10 μg of NADH oxidase, 0.2 mM NADH, 20 μM cytochrome *c*, and 0.3 mM EDTA. In addition, xanthine (final concentration, 0.115 mM) and xanthine oxidase (60 μg of protein) were added to control assays to generate superoxide (13).

Absorption spectra. Absorption spectra (250 to 750 nm) were obtained for pure NADH oxidase at 22°C under anaerobic conditions in sealed semimicro quartz cuvettes. Enzyme (300 to 500 μg in 0.25 ml of 50 mM sodium phosphate buffer [pH 7.0]) and solutions were added to cuvettes within an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) inflated with 85% N_2–10% CO_2–5% H_2. The cuvettes were stoppered and removed from the chamber. Spectral scans were performed on the enzyme under anaerobic conditions before and after reduction by NADH (final concentration, 0.5 mM) and after reoxidation of the enzyme by exposure to oxygen (by removing the cuvette stopper). A Gilford Response II spectrophotometer was used to record absorption spectra.

Alternative electron donors and acceptors. Potential electron donors, α-NADH, α-NADPH, and β-NADPH, were tested in place of β-NADH in the NADH oxidase assay. All were tested at a final concentration of 0.2 mM under standard assay conditions (22°C, 340 nm, 100 to 200 ng of protein from NADH oxidase).

NADH oxidase (350 ng) in anaerobic buffer under 100% nitrogen was assayed with potential electron acceptors other than oxygen. The ability of the enzyme to reduce H_2O_2 (final concentration, 2 mM), $NaNO_2$ (2 mM), $NaNO_3$ (2 mM), and Na_2SO_4 (2 mM) was determined by monitoring the oxidation of NADH at 340 nm. The ability of the enzyme to reduce the following other acceptors was determined at the final concentrations and wavelengths indicated: FAD (50 μM, 450 nm), flavin mononucleotide (50 μM, 450 nm), cytochrome *c* (20 μM, 550 nm), nitroblue tetrazolium (0.5 mM, 560 nm), 2,6-dichlorophenol-indophenol (DCPIP) (0.2 mM, 600 nm), and KFeCN (2 mM, 405 nm).

Chemical inhibitors and stimulators. The following chemicals were tested for their effect on NADH oxidase activity: $CoCl_2$, $NiCl_2$, $AlCl_2$, $BaCl_2$, $ZnCl_2$, $CuSO_4$, $HgCl_2$, $MgSO_4$, $MnCl_2$, $CaCl_2$, EDTA, *p*-chloromercuribenzoic acid, quinacrine, quinine, cyanide, iodoacetate, azide, and arsenate. The enzyme (120 ng) was incubated at 22°C in a 3-ml solution of the compound. The final concentration of most chemicals was 0.5 mM, except that quinacrine, $HgCl_2$, and *p*-chloromercuribenzoic acid were tested at a final concentration of 0.1 mM. After 20 min, NADH was added (final concentration, 0.2 mM), and oxidase activity was measured. The activity of the oxidase in the presence of a particular chemical was compared with that of control assays without added chemical. Other control assays contained NADH and test compound but no enzyme.

Optimum pH. The optimum pH for NADH oxidase was determined by using the following buffers (8, 35): 50 mM sodium phosphate buffer (pH 6.0 to 8.0), 50 mM Tris buffer (Trizma-HCl plus Trizma base [pH 8.0 to 9.5]), and citrate-phosphate buffer (pH 4.5 to 6.0). The molarities of citrate and phosphate in the citrate-phosphate buffers varied depending on pH (8). Oxidase activity was assayed at 22°C by using 100 to 150 ng of oxidase protein. Control assays without added enzyme were used to detect and adjust for spontaneous breakdown of NADH in low-pH buffers.

K_m and V_{max} determinations. K_m and V_{max} values of the *S. hyodysenteriae* NADH oxidase for β-NADH were estimated from Lineweaver-Burk plots (36, 42). Assay mixes used in these determinations contained NADH at final concentrations of 7.0 to 100 μM and 60 ng of NADH oxidase.

Identification of cofactor. An NADH oxidase cofactor was extracted by adding a firefold-larger volume of methanol (HPLC grade) to a solution of the enzyme (approximately 0.1 mg of protein) in 50 mM sodium phosphate buffer (pH 7.0). This solution was heated in a boiling water bath for 15 min, cooled on ice, and centrifuged at 14,000 × *g* for 15 min at 5°C to remove denatured protein. The supernatant was concentrated 10- to 25-fold under a stream of nitrogen gas. Samples (10 μl) of the resulting yellow solution were analyzed by chromatography on thin-layer Silica Gel 60 plates (0.25 mm by 20 cm by 20 cm; order no. 118457; Pierce Chemical Co., Rockford, Ill.). As described by Poole and Claiborne (27), two solvent systems were employed, Na_2HPO_4–$12H_2O$ (5% [wt/vol] in water) and butanol-acetic acid-water (12:3:5). Methanol solutions (0.1 mM) of FAD, flavin mononucleotide, and riboflavin, both alone and mixed with the extracted cofactor, were used as standards. Migration of the compounds was monitored with a UV lamp. Extracted cofactor and all flavin solutions were protected from light whenever possible.

Amino acid sequencing. Purified NADH oxidase was electroblotted onto polyvinylidene difluoride membranes, and the N-terminal amino acids were determined by Edman

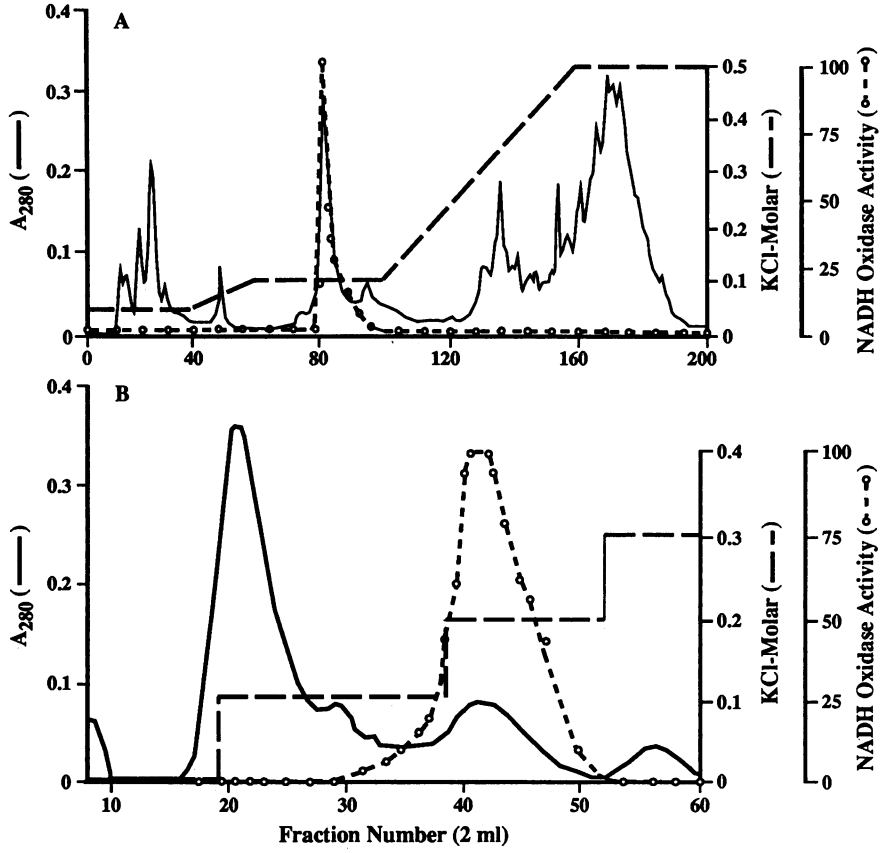

FIG. 1. Purification of *S. hyodysenteriae* NADH oxidase by chromatography. Oxidase activity is expressed as a percentage of the activity in the fraction with the highest activity. Protein elution was monitored at 280 nm. (A) HPLC DEAE-5-PW column. Desalted samples (4 to 10 ml) containing approximately 12 mg of protein precipitated by ammonium sulfate (60 to 80% salt saturation) were injected onto the column. Elution buffer was 10 mM Tris (pH 8.2) at room temperature with increasing concentrations of KCl. The flow rate was 4 ml/min, and the attenuation setting was 64. Fractions (2 ml) from the column were mixed with 2 ml of cold PBCF buffer, and 1- to 20-μl samples were assayed for enzyme activity. (B) Gravity-fed Matrex Gel Blue A agarose column (dye affinity), 5 by 1.6 cm. Desalted fractions (3.5 ml, approximately 5 mg of protein), after ion-exchange chromatography, were deposited onto the column. Elution buffer was 10 mM sodium phosphate (pH 7.0) at 5°C with increasing concentrations of KCl. The flow rate was 0.7 ml/min. Fractions (2 ml) were collected, and 10-μl samples were assayed for enzyme activity.

degradation and HPLC analysis (21) at the Iowa State University Protein Facility, Ames.

Chemicals. All chemicals, enzymes, and reagents were of the highest purity commercially available. Except for those obtained from suppliers mentioned above, all were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

NADH oxidase purification. In early studies, the *S. hyodysenteriae* NADH oxidase in 50 mM phosphate buffer rapidly lost activity. Adding FAD and L-cysteine (PBCF buffer) improved enzyme stability, as reported for other NADH oxidases (12, 16). Nevertheless, there remained an unavoidable and irreversible decrease (20 to 35%) in specific activity when the enzyme was stored at either −80 or 5°C between purification steps.

NADH oxidase was purified from *S. hyodysenteriae* cell lysates by ultracentrifugation, precipitation at high concentrations of $(NH_4)_2SO_4$, and column chromatography (Fig. 1; Table 1). The yellow color of the enzyme was useful for detecting fractions or samples with activity. During purification, enzyme-specific activity increased about 119-fold, with 1% recovery of total enzyme activity (Table 1). All biochemical characteristics of the *S. hyodysenteriae* NADH oxidase were determined for enzyme obtained after size-exclusion chromatography, the final purification step.

Molecular mass and isoelectric pH. Purified enzyme migrated as a single band on denaturing electrophoretic gels and had an estimated molecular mass of 47,000 to 48,000 Da (Fig. 2). Native purified enzyme was eluted just ahead of an ovalbumin protein standard (43 kDa) and behind BSA (66 kDa) on a size-exclusion chromatography column (data not shown). Thus, the *S. hyodysenteriae* NADH oxidase is a monomeric protein with an estimated molecular mass of 47 to 48 kDa.

On an isoelectric focusing gel, purified oxidase separated into three protein bands with NADH-oxidizing activity. These enzymatically active proteins had estimated pI values of 5.25, 5.35, and 5.45.

Absorption spectra and cofactor analysis. An absorption spectrum of the oxidized NADH oxidase had peaks at 274, 376, and 448 nm and a shoulder at 472 nm (Fig. 3). The peak at 448 nm disappeared when NADH was added under anaerobic conditions and reappeared when oxygen was

TABLE 1. Purification of NADH oxidase from *S. hyodysenteriae* B204 cell lysates[a]

Purification stage	Total protein (mg)	Sp act (U/mg)[b]	Total activity (U)	Recovery (%)[c]	Purification factor[d]
Crude cell lysate	8,120	1.4	11,370	100	1
Ultracentrifuged cell lysate (S_1)	2,180	3.3	7,200	63	2.4
60 to 80% $(NH_4)_2SO_4$ precipitate	840	6.1	5,120	45	4.4
Anion-exchange fraction	35	74	2,590	23	53
Dye-affinity fraction	2.4	138	331	3	98
Size-exclusion fraction	0.6	167	100	1	119

[a] NADH oxidase activity was determined by measuring NADH oxidation at 340 nm.
[b] Specific activity is expressed as enzyme units per milligram of protein. One unit equals the amount of enzyme oxidizing NADH at a rate of 1 μmol min^{-1} at 22°C.
[c] Recovery is the total activity expressed as a percentage of the total activity of the crude cell lysate.
[d] Purification factor is the specific activity of the sample divided by the specific activity of the crude cell lysate.

added to the NADH-reduced enzyme (Fig. 3, insert). These spectra are typical for a flavoprotein and other NADH oxidases (16, 24, 32).

When NADH oxidase was denatured in hot methanol, a yellow compound was released, indicating a noncovalent association. This compound fluoresced under a hand-held, short-wavelength UV lamp and migrated with FAD during thin-layer chromatography. Thus, changes in absorption spectra of the enzyme with NADH and oxygen (448 nm; Fig. 3) represent reduction and oxidation of an FAD coenzyme. On the basis of an extinction coefficient of 11.3×10^3 M^{-1} cm^{-1} for FAD at 448 nm (32), the absorbance of oxidized NADH oxidase (300 μg; $A_{448} = 0.262$), and a molecular mass of 47 kDa (Fig. 2), 23 nmol of FAD was associated with about 25 nmol of purified enzyme. Thus, the *S. hyodysenteriae* NADH oxidase contains an estimated 1 mol of FAD per mol of protein.

pH optimum. *S. hyodysenteriae* NADH oxidase was active at a pH between 5.0 and 9.5. Optimum activity (90 to 100% relative activity) of the enzyme occurred at a pH of 5.5 to 7.0. Morpholinoethanesulfonic acid (MES) buffer (50 mM, pH 5.5 to 6.5) inhibited oxidase activity, and acetate buffer (50 mM) at pH 5.5 slightly stimulated oxidase activity.

NADH oxidase products. Only trace amounts (<2 nmol) of H_2O_2 were detected in assays in which 500 nmol of NADH was oxidized by the enzyme. These trace amounts could represent intermediate products (H_2O_2 or another peroxide) of the enzyme. Added hydrogen peroxide (2.5, 5, or 50 nmol) was detected after the NADH oxidase assay was performed, an indication that the enzyme did not have catalase activity and that H_2O_2 generated by the oxidase would have been detected had it been a significant product. During oxidation of NADH by NADH oxidase, superoxide was not detected by a cytochrome *c* reduction assay. In parallel control experiments, the addition of a superoxide-generating system (xanthine plus xanthine oxidase [13]) to the NADH oxidase assay resulted in rapid cytochrome *c* reduction. These results indicate that H_2O_2 and superoxide were not produced in sufficient amounts to be considered end products. They are indirect evidence that water is produced from the reduction of oxygen by the *S. hyodysenteriae* enzyme.

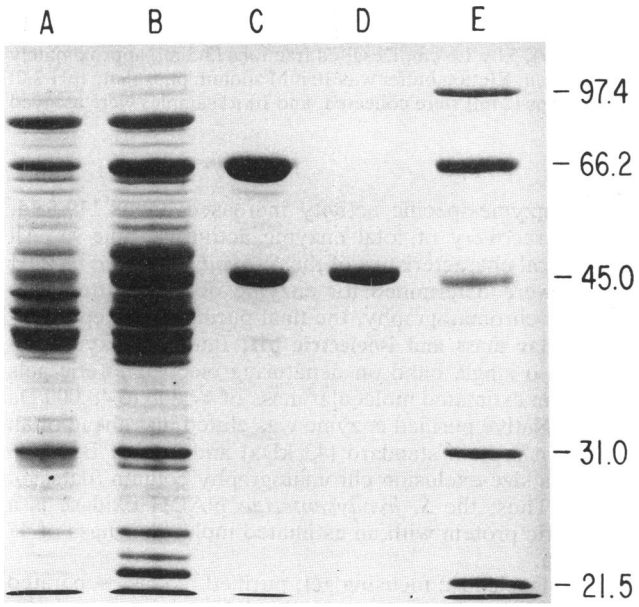

FIG. 2. Electrophoresis of proteins from various stages of *S. hyodysenteriae* NADH oxidase purification. Proteins were separated on an SDS-10% polyacrylamide gel and were stained with Coomassie blue. Lanes: A, S_1 fraction (15 μg of protein); B, ammonium sulfate 60 to 80% precipitate (7 μg); C, HPLC anion-exchange fraction (5 μg); D, HPLC size-exclusion fraction (1 μg); E, molecular mass standards (4 μg). Molecular masses (in kilodaltons) of standard proteins are given on the right.

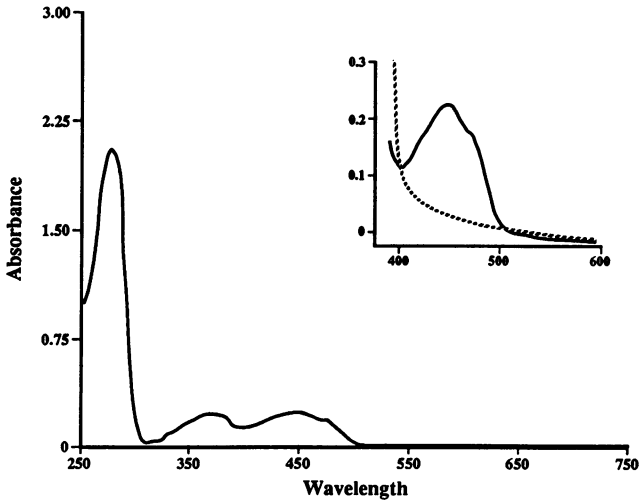

FIG. 3. Absorption spectra of *S. hyodysenteriae* NADH oxidase. Purified NADH oxidase (350 μg of protein) was dissolved in 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0) and scanned at 22°C. Inset: absorption spectra of reduced enzyme (300 μg) 3 min after addition of NADH (0.5 mM) under anaerobic conditions (····) and oxidized enzyme 8 min after exposure to air (8——).

TABLE 2. Comparison of NADH oxidases from *S. hyodysenteriae*, *E. faecalis*, and *T. thermophilus*[a]

Bacterium	NADH oxidase characteristic					
	Sequence (N terminus)	Mol mass (kDa)	Subunits	Isoelectric pH	K_m (μM)[b]	Product
S. hyodysenteriae B204	MKVIVIGCNHAGTWAAK	47	1	5.2–5.4	10	H_2O
E. faecalis ATCC 11700 and 10C1	MKVVVVGCTHAGTSAVK	51 (48.9)[c]	1[c]	5.2 (4.7)	40	H_2O
T. thermophilus HB8	MEATLPVLDAKTAALKR	27	1	ND	4	H_2O_2

[a] References: *S. hyodysenteriae* (present article); *E. faecalis* (12, 31, 34); *T. thermophilus* (24, 25). The information in the table is based on results with purified protein or deduced from the nucleotide sequence of the cloned gene (results from the latter shown as values in parentheses). ND, not determined.

[b] K_m values represent β-NADH concentrations.

[c] The *E. faecalis* NADH oxidase was described as a monomer with a molecular mass of 51 kDa by Schmidt et al. (34). Ahmed and Claiborne (2) reported that the enzyme was a dimer of identical 51.5-kDa subunits.

Alternative electron donors and acceptors. With oxygen as an electron acceptor, α-NADPH and β-NADPH were not detectably oxidized by the NADH oxidase (<0.5% relative activity of β-NADH). α-NADH gave 0.7% of the activity of β-NADH. These results indicate that β-NADH is the natural electron donor for the *S. hyodysenteriae* NADH oxidase.

The NADH oxidase did not have detectable dehydrogenase or diaphorase activities. That is, nitrate, nitrite, sulfate, flavin mononucleotide, FAD, cytochrome *c*, nitroblue tetrazolium, and ferricyanide at the concentrations tested were not reduced under anaerobic assay conditions. DCPIP was reduced at the same rate with or without enzyme. NADH oxidase activity with H_2O_2 as the oxidizing agent either was not detected or was at the limit of detection (≤1.3 μmol of NADH oxidized min^{-1} mg^{-1}).

K_m and V_{max} for β-NADH. On the basis of Lineweaver-Burk plots constructed from the data of three experiments, the apparent K_m and V_{max} values of the *S. hyodysenteriae* NADH oxidase for β-NADH were 10 μM (range, 9 to 12 μM) and 110 μmol min^{-1} mg^{-1} (range, 90 to 160 μmol min^{-1} mg^{-1}), respectively.

Chemical inhibitors. EDTA did not inhibit activity, and added $MgSO_4$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $NiCl_2$, $AlCl_2$, $BaCl_2$, or $ZnCl_2$ did not enhance activity, suggesting that the NADH oxidase does not require a metal cofactor. Heavy-metal ions Cu^{2+} and Hg^{2+} strongly inhibited NADH oxidase (90 and 100% inhibition, respectively). Spectrophotometer cuvettes had to be soaked in concentrated nitric acid to remove inhibitory traces of Hg^{2+}. Enzyme activity was reduced 15 and 60% by quinine and quinacrine (inhibitors of flavin enzymes), respectively, and also by cyanide (35%). The oxidase was strongly inhibited (98 to 100% reduction in activity) by the sulfhydryl active compounds $HgCl_2$, *p*-chloromercuribenzoic acid and iodoacetate.

N-terminal amino acid sequence. The amino acid sequence at the N-terminal end of the *S. hyodysenteriae* NADH oxidase is given in Table 2. Only one sequence was obtained, an indication that the purified enzyme was homogeneous. Cysteines in the NADH oxidase could not be detected since they had not been modified by pyridylethylation prior to sequencing of the enzyme. Consequently, Cys-8 was deduced from the nucleotide sequence of the oxidase gene (38a).

DISCUSSION

S. hyodysenteriae B204 cells contain high levels of NADH oxidase activity (38). Experimental results reported in this article indicate that this oxidase is a soluble (non-membrane-associated) monomeric protein with a molecular mass of 47 to 48 kDa. The enzyme has spectral properties and inhibitor sensitivities typical of a flavoprotein. FAD is noncovalently bound to the enzyme at a molar ratio of 1:1. The product of the *S. hyodysenteriae* NADH oxidase reaction is water. Indirect evidence for this conclusion is the finding that H_2O_2 and superoxide are not produced in significant amounts during NADH oxidation by the enzyme. Further evidence is that the *S. hyodysenteriae* oxidase resembles the NADH oxidase of *Enterococcus faecalis* in biochemical properties (discussed below) and is likely to carry out the same reactions. The *E. faecalis* enzyme forms water from a four-electron reduction of oxygen (12, 34).

The *S. hyodysenteriae* NADH oxidase separated into three bands of activity (subforms) on an isoelectric focusing gel. These bands are unlikely to represent different enzymes since only one N-terminal amino acid sequence was obtained when purified NADH oxidase was sequenced (Table 2). Additionally, we have detected only one copy of the NADH oxidase gene in restriction digests of the *S. hyodysenteriae* genome (38a). The different subforms could represent different redox states of the enzyme. A proposed scheme for the activity of the *E. faecalis* NADH oxidase includes intermediate redox states in the functioning of that enzyme (3).

A number of bacterial NADH oxidases have been characterized (4, 12, 15, 16, 20, 24, 29, 32, 34), and those from *E. faecalis* and *Thermus thermophilus* have been sequenced (25, 31). Ahmed and Claiborne (2, 3) and Ross and Claiborne (31) have extensively characterized the *E. faecalis* enzyme and related various functions of the oxidase to its primary structure. In biochemical properties, the NADH oxidase of *S. hyodysenteriae* closely resembles the NADH oxidase of *E. faecalis*, and both of these enzymes are distinct from that of *T. thermophilus* (Table 2).

The biochemical similarities between the *S. hyodysenteriae* and the *E. faecalis* NADH oxidases also include similarities in primary structure (Table 2) (38a). The N-terminal end of the *S. hyodysenteriae* NADH oxidase has 12 of 17 amino acids identical with those of the *E. faecalis* oxidase (Table 2). In this region, the *S. hyodysenteriae* sequence G_7CNHAG corresponds to the *E. faecalis* sequence G_7CTHAG, a variation of the glycine triad GXGXXG associated with FAD binding (31, 45). The His-10 is conserved in both the NADH oxidase and the NADH peroxidase of *E. faecalis* and has been proposed as an active-site base, essential for activity of both enzymes (30, 31, 41). Additionally, the translated sequence of the *S. hyodysenteriae* NADH oxidase gene indicates a cysteine at position 42 of the enzyme (38a). The Cys-42 is present in both the NADH oxidase and NADH peroxidase of *E. faecalis* and represents a redox center essential for enzyme activity (28, 30, 31). A comparison of the complete amino acid sequences of the *S. hyodysenteriae* and *E. faecalis* NADH oxidases will likely

provide additional insight into the functioning and, possibly, the evolution of this enzyme.

S. hyodysenteriae, a gram-negative, anaerobic spirochete, and *E. faecalis*, a gram-positive, facultatively anaerobic coccus, are phylogenetically distinct species. Nevertheless, both species inhabit the mammalian intestinal tract. The apparent resemblance between their NADH oxidases is intriguing, a suggestion, perhaps, of horizontal gene transfer and an indication that NADH oxidase could be more ubiquitous among gastrointestinal bacteria than is now appreciated.

What role does NADH oxidase play in the physiology and ecology of *S. hyodysenteriae*? Studies determining the influence of oxygen on the growth and metabolism of *S. hyodysenteriae* might answer this question. NADH oxidase could be an alternative mechanism for NADH oxidation and recycling, allowing *S. hyodysenteriae* cells to gain ATP by converting acetyl coenzyme A to acetate instead of butyrate (38). However, in this role, NADH oxidase appears redundant since *S. hyodysenteriae* cells also contain an NADH-ferredoxin oxidoreductase pathway for oxidizing NADH generated by enzymes of the Embden-Meyerhof-Parnas pathway (38). Alternatively, or additionally, NADH oxidase could act as a defense mechanism against oxidative stress, enabling this anaerobic pathogen to survive among oxygen-respiring and oxygen-carrying host tissues. If this hypothesis were correct, oxidase-deficient strains of *S. hyodysenteriae* would be more sensitive to oxygen and could be less virulent for swine than wild-type strains.

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