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Characterization of the Respiratory NADH Dehydrogenase of *Escherichia coli* and Reconstitution of NADH Oxidase in *ndh* Mutant Membrane Vesicles†

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ABSTRACT: Highly purified preparations of the cholate-solubilized respiratory NADH dehydrogenase, isolated from genetically amplified *Escherichia coli* strains [Jaworowski, A., Campbell, H. D., Poulis, M. I., & Young, I. G. (1981) *Biochemistry 20*, 2041–2047], have been characterized. Enzyme preparations were shown to contain 70% (w/w) lipid, predominantly phosphatidylethanolamine. One mol of noncovalently bound FAD and ~1 mol of ubiquinone/mol of enzyme subunit were detected. The purified enzyme was shown to contain only low levels of Fe and acid-labile S, indicating the absence of iron–sulfur clusters. No Cu, Mo, W, or covalently bound P was detected, and no evidence for other chromophores was obtained from visible and ultraviolet absorption spectra of the purified enzyme or of the delipidated polypeptide prepared by gel filtration in sodium dodecyl sulfate. Protein chemical studies verified that the enzyme consists of a single polypeptide species of M_r 47 000, and the N- and C-terminal cyanogen bromide peptides were identified. The pure enzyme was shown to reconstitute membrane-bound, cyanide-sensitive NADH oxidase activity in membrane vesicles prepared from *ndh* mutant strains.

The oxidation of NADH via the respiratory chain with concomitant generation of energy is one of the primary features of aerobic respiration. In *Escherichia coli*, the enzyme catalyzing the first step in this pathway, the respiratory NADH dehydrogenase, is present in the cytoplasmic membrane together with the other components of the electron transport chain.

The low levels of this enzyme in wild-type cells, and the presence of high levels of contaminating NADH dehydrogenase activities, have hampered its isolation and characterization (Dancey et al., 1976; Gutman et al., 1968; Bragg & Hou, 1967a,b). The recent cloning of the *E. coli* respiratory NADH dehydrogenase structural gene (Young et al., 1978), however, has allowed several new approaches to be used to study this enzyme. Amplification of enzyme levels in vivo to 100 times those found in the wild type has enabled it to be purified for the first time with high catalytic activity toward ubiquinone as electron acceptor and in quantities permitting detailed characterization (Jaworowski et al., 1981). The purified enzyme shows a single band of apparent molecular weight 45 000 on $NaDodSO_4$[1] gel electrophoresis. DNA sequencing (Young et al., 1981) has revealed the complete primary structure of the enzyme, which consists of 433 amino acid residues, with a molecular weight of 47 200. By use of the cloned DNA as template, a catalytically active, membrane-bound form of the enzyme has been produced in vitro in a cell-free, coupled transcription–translation system (Poulis et al., 1981).

We describe here the characterization of the purified cholate-solubilized enzyme preparation and show that the enzyme retains the ability to reconstitute functional NADH oxidase activity in membrane particles prepared from *ndh* mutant strains.

Experimental Procedures

Bacterial Strains and Plasmids. The *ndh* mutant strain IY12, a derivative of *E. coli* K12, has been described previously (Young et al., 1978). Plasmid pIY1 (Young et al., 1978) possesses the 2500 base pair *ndh* fragment cloned into the *Eco*RI site of pSF2124 (So et al., 1975). Plasmid pLJ3 (Johnsrud, 1978) carries a double *lac* promoter fragment at the *Eco*RI site of pMB9 (Rodriguez et al., 1976). Plasmid pIY10 is a derivative of pMB9, carrying both the *ndh* and

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[1] Abbreviations used: *ndh*, structural gene for NADH dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; ubiquinone-*n*, ubiquinone isoprenologue containing *n* isoprene units in the side chain; fluorescamine, 4-phenylspiro[furan-2(3*H*)-1′-phthalan]-3,3′-dione; EDTA, ethylenediaminetetraacetic acid disodium salt; TPCK, L-1-(*p*-toluenesulfonyl)-amido-2-phenylethyl chloromethyl ketone.

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double *lac* promoter fragments at the *Eco*RI site, and was constructed as follows.

Plasmids pIY1 and pLJ3 were digested with *Eco*RI endonuclease, ligated, and transformed into strain IY12 as described previously (Jaworowski et al., 1981). Selection of transformants carrying the *ndh* gene recloned into pMB9 was made on mannitol-minimal plates (Young & Wallace, 1976) containing tetracycline (10 μg mL^{-1}). Viable colonies were then tested for the presence of hybrid plasmids containing the *lac* fragment by plating onto mannitol-minimal plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Jaworowski et al., 1981). Strains with multicopy plasmids carrying the *lac* promoter produce blue colonies on this medium (Johnsrud, 1978). Membrane particles prepared from three such clones were assayed for NADH:ubiquinone oxidoreductase activity (see below). The clone with the highest specific activity was designated IY91, and the plasmid it contains, pIY10.

Preparation of NADH Dehydrogenase. The respiratory NADH dehydrogenase was prepared essentially as described elsewhere (Jaworowski et al., 1981), except that when strain IY91 was used, the chloramphenicol amplification step was omitted, and the bacteria were simply grown to late exponential phase. Membranes particles (200 mL) obtained from two 90-L growths of cells were washed, solubilized, and chromatographed on a 5 × 30 cm column of hydroxylapatite as described, except that the solubilized material was centrifuged for 2.5 h at 60000 rpm in a Beckman 60Ti rotor to give better pelleting of insoluble material. In this way, 200 mg of enzyme protein, with an NADH:ubiquinone oxidoreductase specific activity of 530 units mg^{-1}, was obtained. Enzyme solutions were concentrated by ultrafiltration using an Amicon TCF10 ultrafiltration apparatus fitted with a PM10 membrane. Enzyme preparations were frozen in liquid N_2 and stored at −60 °C.

Protein Determination. Protein was determined by using the method of Lowry et al. (1951) as described elsewhere (Jaworowski et al., 1981). For conversion to absolute protein concentrations, values obtained with the purified enzyme were multiplied by 0.88, the correction factor determined by amino acid analysis (see below). The molarity of solutions with respect to the enzyme subunit was calculated by using the molecular weight for the processed, mature enzyme of 47200, calculated from the predicted amino acid sequence (Young et al., 1981).

Enzyme Assays. NADH:ubiquinone oxidoreductase and NADH oxidase activities were determined spectrophotometrically as described elsewhere (Jaworowski et al., 1981).

Extraction and Estimation of Lipid. Purified NADH dehydrogenase preparations (~5 mg of protein/experiment) were dialyzed for at least 24 h at 4 °C against three changes of distilled water (each 40 volumes). The solution was freeze-dried, resuspended in 5 mL of distilled H_2O, and extracted 5 times with an equal volume of $CHCl_3$–CH_3OH, 2:1 (v/v). The lipid extract was extracted once with 2 mL of H_2O and then analyzed.

Phospholipid P was estimated by measuring the enhancement of rhodamine 6G fluorescence (Schiefer & Neuhoff, 1971) with pure *E. coli* phosphatidylethanolamine (Sigma) as the standard. Dry weight and total phosphorus determinations were performed on lipid extracts after evaporation of the solvent and drying in vacuo to constant weight.

Chromatography of extracted lipids was carried out on silica gel thin-layer plates (Merck; F_{254}; 0.5 mm thick) by using $CHCl_3$–CH_3OH–H_2O, 65:31:4 (by volume), and $CHCl_3$–CH_3OH–aqueous NH_3 (sp gr 0.88), 65:30:5 (by volume), as solvents (Ames, 1968). Lipids were generally visualized with iodine vapor, and those with free amino groups by spraying with ninhydrin (2 g L^{-1} in acetone), followed by heating.

Estimation of Ubiquinone. Concentrated enzyme preparations (5 mg of protein mL^{-1}) were extracted by the method of Wallace & Young (1977), and ubiquinone was estimated by reduction with borohydride as described elsewhere (Crane & Barr, 1971). The recovery obtained with added ubiquinone-8 (55%) was used to correct the measured values. Thin-layer chromatography of the extracts was performed as described previously (Wallace & Young, 1977). Ubiquinone was also estimated after alkaline hydrolysis in the presence of 1,2,3-benzenetriol (Hatefi, 1959).

Estimation of Flavin. Enzyme (20 mL; 0.3 mg mL^{-1}; NADH:ubiquinone oxidoreductase specific activity 530 units mg^{-1}) was dialyzed at 4 °C against 90 volumes of 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% (w/v) cholate, for 24 h with one change of dialysis buffer. The flavin content of the dialyzed enzyme was determined fluorometrically according to Udenfriend (1962) and corrected for the measured flavin concentration of the final dialysate (~2% correction). Trichloroacetic acid precipitated protein was removed by filtration through 0.4-μm Millipore filters.

Flavin extracts were chromatographed on silica gel thin-layer plates (Merck; F_{254}; 0.5 mm thick) with 1-butanol–acetic acid–H_2O, 12:3:5 (by volume), as the solvent (Fazekas & Kokai, 1971).

Metal Analyses. Concentrated enzyme samples were dialyzed at 4 °C against 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% (w/v) cholate. Aliquots were wet-ashed by the method of Beinert (1978). Fe and Cu were determined as described by Beinert (1978), except that the volume of organic solvent containing chelator used for extraction was increased to 1 mL. After wet-ashing with H_2SO_4 and H_2O_2 as above, Mo and W were determined according to Cardenas & Mortenson (1974), except that the volume of isopentyl acetate was reduced to 2 mL.

Iron was also determined without ashing by the total Fe procedure of Doeg & Ziegler (1962). For assays on column fractions, this procedure was scaled up 4-fold, except that 1 mL of bathophenanthroline in isopentyl alcohol was still used for extraction.

Sulfide Determination. Inorganic sulfide was measured by a slight modification of the methods of Rabinowitz (1978) and King & Morris (1967). Protein samples were incubated with the alkaline zinc reagent for at least 30 min (Suhara et al., 1975). By minimization of the loss of H_2S, an ϵ_{app} value of 35000 M^{-1} cm^{-1} was obtained (Gustafsson, 1959) by using iodometrically standardized Na_2S solutions prepared under O_2-free conditions (King & Morris, 1967).

Phosphate Determination. Purified enzyme was treated with $NaDodSO_4$ and 2-mercaptoethanol and chromatographed on Sephacryl S-300 equilibrated with 0.05 M NH_4HCO_3 and 1% (w/v) $NaDodSO_4$, as described elsewhere (Poulis et al., 1981). Samples of the enzyme polypeptide in $NaDodSO_4$ solution (500 μL, ~0.2 mg mL^{-1}) were ashed with H_2SO_4 and H_2O_2 as for metal analyses. The following procedure, based on reported methods (Chen et al., 1956; Ames & Dubin, 1960; Bloch & Schlesinger, 1973), was used to determine the phosphate content. To the ashed sample was added 1 mL of ascorbic acid solution (28 g L^{-1}) with rapid mixing. A solution of ammonium molybdate tetrahydrate (7.2 g L^{-1}; 1 mL) was immediately added with further mixing. The tubes were incubated at room temperature for at least 4 h, or at 37 °C for

60 min, and then the absorbance at 820 nm was measured. Blanks and standards (0–25 nmol of KH_2PO_4 in 500 μL of 0.05 M NH_4HCO_3 and 1% $NaDodSO_4$) were ashed and treated as above. Excessive loss of H_2SO_4 during ashing was avoided to ensure that the final concentration of H_2SO_4 remained within the range of 0.5–1.0 N (Chen et al., 1956).

Spectral Measurements. Visible and ultraviolet absorption spectra were recorded by using a Cary 118C recording spectrophotometer. Fluorescence measurements were made by using a Perkin-Elmer Model 3000 spectrofluorometer.

^{55}Fe Incorporation. Mineral salts growth medium (Stroobant et al., 1972) was freed of Fe by aging and Millipore filtration (Rosenberg, 1979). All glassware was cleaned with HNO_3 and soaked in 50 mM EDTA. The medium was supplemented with mannitol, amino acids, and thiamine but not casamino acids, as described elsewhere (Jaworowski et al., 1981). To 5 L of medium was added 1 mCi of $^{55}FeCl_3$ (Amersham; 253 Ci mol^{-1}) in 0.1 N HCl, plus sufficient nonradioactive Fe to give the usual final Fe concentration (3.6 μM). Strain IY91 was grown to late exponential phase on this medium in 5 × 1 L batch cultures, cells were harvested (yield, 11.4 g wet weight), and membranes were prepared and washed (Jaworowski et al., 1981; Wallace & Young, 1977). The membranes were then solubilized with cholate–KCl and chromatographed on a column (1.6 × 12 cm) of hydroxylapatite (Jaworowski et al., 1981). Fractions were assayed for protein and NADH:ubiquinone oxidoreductase activity. The ^{55}Fe content of fractions was measured by liquid scintillation counting with a Packard Tri-Carb 460CD liquid scintillation spectrometer by using a xylene–Triton X-100 based scintillant.

Amino Acid Analysis. Purified NADH dehydrogenase was dialyzed at 4 °C against 3 × 40 volumes of 5 mM potassium phosphate buffer, pH 7.5, and then 2 × 40 volumes of glass-distilled H_2O (10–12 h/change). Aliquots were hydrolyzed in 6 N HCl in vacuo for 22, 48, and 96 h at 110 °C. Aliquots were also taken for protein determinations by the method of Lowry et al. (1951) and the remainder was freeze-dried. Amino acid analysis was performed on a modified Beckman 120C amino acid analyzer fitted with a single column (6 mm) of W2 resin (Beckman Instruments Inc., Palo Alto, CA). Peaks were integrated with a Beckman 126 data system. The results of amino acid analysis showed that protein concentration values obtained by the method of Lowry et al. (1951) for purified enzyme using bovine serum albumin as the standard should be multiplied by 0.88 to give the true protein concentration.

Delipidation of the freeze-dried material was carried out at 4 °C as follows. By use of a vortex mixer, 53 mg of solid was extracted with 6 × 4 mL of $CHCl_3$–CH_3OH, 2:1 (v/v), chilled to −15 °C. The protein was collected by centrifugation in a bench centrifuge. The final pellet was dried under N_2, washed twice in ice-cold distilled H_2O, and desiccated under vacuum. This material, with and without performic acid oxidation (Hirs, 1956), was hydrolyzed for 22 h and analyzed as above.

Peptide Mapping of NADH Dehydrogenase. Solvent-delipidated NADH dehydrogenase (6.6 mg) was oxidized with performic acid (Hirs, 1956). The oxidized protein was resuspended in 4 mL of 0.063 M NH_4HCO_3 and then digested for 4 h at 37 °C by using 2% (relative to the weight of oxidized protein) TPCK-treated trypsin (Worthington). After removal of the insoluble "core" material, the soluble tryptic peptides were separated by peptide mapping on Whatman 3MM paper (Bell et al., 1968). The first dimension was electrophoresis at pH 4.7 (40 V cm^{-1}; 1.5 h) and the second dimension was ascending chromatography (18 h) in 1-butanol–acetic acid–pyridine–H_2O, 15:3:12:10 (by volume). Peptides were visualized with ninhydrin (2 g L^{-1} in acetone). Alternatively, maps were sprayed with a solution of fluorescamine (Hoffmann-La Roche; 2 mg L^{-1} in acetone containing 6 mL of pyridine L^{-1}), and the peptides viewed under UV light (Lai, 1977). The peptides were extracted by macerating the cut-out spots in 6 N HCl, which was then filtered. The resulting solution was hydrolyzed for 22 h and analyzed as described above. When further purification was required, peptides were cut out on strips (2 × 12 cm), which were sewn onto fresh sheets of Whatman 3MM paper, and electrophoresed (40 V cm^{-1}; 1 h) in a third dimension at pH 1.9 (Bell et al., 1968).

CNBr Peptides. NADH dehydrogenase preparations were dialyzed and delipidated as described above. Five milligrams of protein was subjected to CNBr cleavage in 70% formic acid for 20 h at room temperature at a CNBr:protein ratio of 1:1 by weight (Gross, 1967). The more soluble of the resulting peptides were separated by three-dimensional peptide mapping as described above. Peptides were visualized with fluorescamine, eluted, and analyzed (see above).

Reconstitution of NADH Oxidase Activity in ndh Mutant Membrane Particles. In method 1, membrane particles (IY12, 50 μL) were placed in glass vials at 0–4 °C, and varying amounts of purified NADH dehydrogenase (0–100 μL; ~30 units mL^{-1}) were added and mixed. Aliquots (5 μL) of the mixture were assayed for NADH oxidase activity in an assay system consisting of 1 mL of 50 mM Tes buffer, pH 7.5, containing 40 μM FAD and 250 μM NADH, preequilibrated at 30 °C. The reaction was started by the addition of reconstituted membrane particles and followed spectrophotometrically at 340 nm. The rate of NADH oxidation was calculated by using $\Delta\epsilon = 6220\ M^{-1}\ cm^{-1}$. In method 2, 1 mL of the above assay mixture, equilibrated at 30 °C, and 5 μL of *ndh* mutant membrane particles were placed in a 1-mL quartz cuvette, various volumes of enzyme (0–100 μL; ~30 units mL^{-1}) were added immediately, and NADH oxidation was followed as above. In both cases, the rates of NADH oxidation obtained were corrected for the low NADH oxidase activities of the mutant membrane particles and the pure enzyme, measured individually in the above assay system. The cyanide sensitivity of the various NADH oxidase activities was determined from the decrease in the rate after addition of 3 μL of 1 M KCN.

Results

Purification of NADH Dehydrogenase. An improved procedure which was more convenient for large-scale preparations than the previous method using chloramphenicol amplification (Young et al., 1978; Jaworowski et al., 1981) was used to increase the levels of the respiratory NADH dehydrogenase in vivo. This involved the construction of a new hybrid plasmid, pIY10, which is derived from pMB9 (Rodriguez et al., 1976) and carries a double *lac* promoter fragment (Johnsrud, 1978) adjacent to the *ndh* gene (see Experimental Procedures). The plasmid pMB9 has a higher copy number than pSF2124 (Helling & Lomax, 1978), the vector previously used (Young et al., 1978). This, together with the effect of the strong *lac* promoter on gene expression (Jaworowski et al., 1981), gave adequate enzyme levels in IY91, the strain carrying pIY10, for successful purification without chloramphenicol amplification. The NADH:ubiquinone oxidoreductase specific activity of membrane particles prepared from IY91 grown on a 9 × 10 L scale and harvested in late exponential phase was consistently ~50 units mg^{-1} (~100-fold above wild-type levels).

Membrane particles were isolated and solubilized with cholate, and the respiratory NADH dehydrogenase was purified by chromatography on hydroxylapatite as described elsewhere (Jaworowski et al., 1981). The preparations obtained were essentially pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis and rechromatography on hydroxylapatite. Protein chemical studies such as the determination of the N-terminal sequence by automated Edman degradation (Young et al., 1981) also failed to detect any major contaminants.

For some purposes, e.g., protein chemical studies, where it was necessary to free the polypeptide from lipid and noncovalently bound prosthetic groups, the enzyme was further purified by column chromatography on Sephacryl S-300 in the presence of $NaDodSO_4$ (Poulis et al., 1981).

Lipid Content of Purified Enzyme. Several independent assays were used to estimate the lipid content of NADH dehydrogenase preparations, following extraction of such preparations with chloroform–methanol, 2:1 (v/v).

The lipid content determined gravimetrically by drying the solvent extract to constant weight was estimated to be 2.2 g of lipid/g of protein, or ~70% by weight. Fluorometric assays using *E. coli* phosphatidylethanolamine as the standard gave a value of 2.1 g of phospholipid/g of protein, which indicates that the major part of the lipid present is phospholipid. The results of total phosphorus analysis, 0.085 g of P/g of protein, were consistent with this interpretation, corresponding to 1.9 g of phospholipid/g of protein assuming an average phospholipid molecular weight of 691 (that of dipalmitoylphosphatidylethanolamine).

Chromatography of chloroform–methanol extracts on silica gel resolved one major and several minor species. Comparison with pure standards, and a positive reaction with ninhydrin, indicated that the major species is phosphatidylethanolamine. The minor species correspond in their mobilities in the solvent system employed to phosphatidylglycerol, cardiolipin, and neutral lipid (Ames, 1968) but were not further characterized.

Ubiquinone Determination. Since it is likely that ubiquinone-8 is the electron acceptor for the respiratory NADH dehydrogenase in vivo, it was of interest to determine whether the purified enzyme contains ubiquinone. Spectrophotometric determinations of ubiquinone in solvent extracts from two different enzyme preparations gave levels of 0.67 and 1.1 mol/mol of enzyme subunit. Similar levels were obtained after alkaline hydrolysis in the presence of 1,2,3-benzenetriol (Hatefi et al., 1979). The identity of the ubiquinone was confirmed by cochromatography with authentic ubiquinone-8 on silica gel (Wallace & Young, 1977).

Flavin Determinations. In preliminary experiments it was found that if FAD was omitted from column buffers during chromatography of the solubilized enzyme on hydroxylapatite, the resulting preparation was inactive unless FAD was present in the assay. On the basis of these findings, 20 μM FAD was routinely included in the column equilibration and gradient buffers. Maximum reactivation of such flavin-depleted preparations was obtained with ~50 μM FAD in the assay (Figure 1). The requirement for FAD was not satisfied by FMN at comparable concentrations. When the enzyme was purified in the presence of FAD, the activity was not dependent upon the presence of flavin in the assay even when such preparations were dialyzed in the absence of flavin for periods as long as 72 h. These results suggest the presence of noncovalent, tightly bound FAD as a prosthetic group.

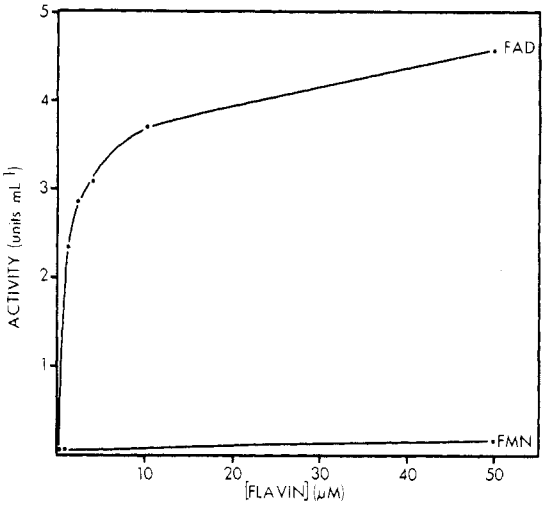

FIGURE 1: Reactivation of flavin-depleted *E. coli* NADH:ubiquinone oxidoreductase by FAD. NADH:ubiquinone oxidoreductase was purified on a small scale exactly as described (Jaworowski et al., 1981), except that FAD was omitted from the hydroxylapatite column equilibration and gradient buffers. Column fractions were assayed under the standard assay conditions (40 μM FAD). The pooled enzyme peak was then assayed for NADH:ubiquinone oxidoreductase activity under the standard assay conditions, except that FAD or FMN was present at the indicated concentration. The activity of the pooled enzyme in the absence of flavin was 0.027 unit mL^{-1}. In the presence of 50 μM FAD, the activity was 7.82 units mL^{-1} (specific activity 130 units mg^{-1}).

Enzyme preparations were dialyzed to remove FAD present in the elution buffer, and then acid-extractable flavin was estimated fluorometrically. A value for total flavin of 1.0 ± 0.1 mol/mol of subunit was obtained. From the relative fluorescence before and after hydrolysis, it was estimated that 67% of this flavin was FAD. Chromatography of flavin extracts on silica gel thin-layer plates, using 1-butanol–acetic acid–water, 12:3:5 (by volume), as the solvent, confirmed that FAD was the major flavin derivative present. Several minor spots, which did not correspond to either FMN or riboflavin, were also observed. These components were not further identified, but it is possible that they are breakdown products derived from FAD.

The protein did not fluoresce in acid solution after $NaDodSO_4$ gel electrophoresis, suggesting the absence of covalently bound flavin (Ohishi & Yagi, 1979; Weiner & Dickie, 1979). This was confirmed by the absorption spectrum of the polypeptide in $NaDodSO_4$ solution and the absence of covalently bound phosphate in the enzyme (see below).

Visible and Ultraviolet Absorption Spectra. The major features of the visible and near ultraviolet spectrum of the purified enzyme are the peaks at 370 and ~440 nm characteristic of FAD in the oxidized form (Figure 2). The apparent absorption coefficient at 450 nm is 14 800 M^{-1} cm^{-1}, with the major contribution to the absorption at this wavelength coming from the flavin prosthetic group (ϵ = 11 300 M^{-1} cm^{-1}; Whitby, 1953). The ultraviolet spectrum (Figure 2b) shows an absorption maximum at 268 nm (ϵ = 98 000 M^{-1} cm^{-1}) which can be attributed to absorption by FAD (ϵ = 38 000 M^{-1} cm^{-1}) and lipid (apparent absorption coefficient 24 000 M^{-1} cm^{-1}; see Figure 2a), as well as the polypeptide itself (ϵ = 23 500 M^{-1} cm^{-1}, based on the tryptophan and tyrosine content). In both the visible and ultraviolet regions of the spectrum there appears to be a contribution from light scattering, presumably due to the particulate nature of the preparation.

The ultraviolet spectrum of the enzyme polypeptide, obtained by gel filtration of the purified enzyme in the presence of $NaDodSO_4$, is that of a typical protein, showing an ab-

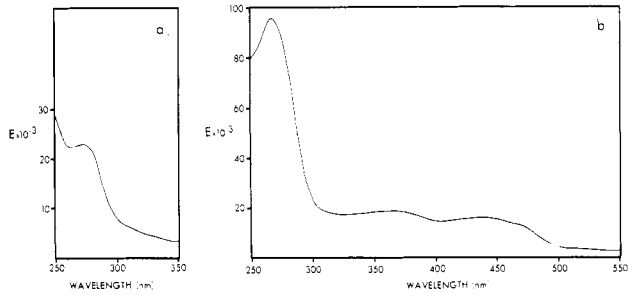

FIGURE 2: Electronic absorption spectra of highly purified *E. coli* NADH dehydrogenase and enzyme-associated lipid. Enzyme from hydroxylapatite chromatography (specific activity 530 units mg^{-1}) was dialyzed at 4 °C against 90 volumes of 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% cholate, for 24 h with one change of dialysis buffer. The protein concentration after dialysis was 0.168 mg mL^{-1}. Panel b shows the electronic absorption spectrum of the dialyzed enzyme solution. A 2-mL aliquot of dialyzed enzyme was extracted with 3 × 2 mL of $CHCl_3$–CH_3OH, 2:1 (v/v). The lipid extract was rotary evaporated and then redissolved in 2 mL of absolute ethanol. Panel a shows the ultraviolet absorption spectrum of the lipid extract in ethanol. Apparent molar absorption coefficients (E) were calculated relative to the molarity of the protein (obtained as described under Experimental Procedures).

sorption maximum at 277 nm and a shoulder at 288 nm attributable to the presence of tryptophan. Such preparations exhibited no absorption in the visible region, confirming that there is no covalently bound flavin present.

Iron and Acid-Labile Sulfide Determinations. Fractions across the NADH:ubiquinone oxidoreductase peak obtained by large-scale hydroxylapatite chromatography (Jaworowski et al., 1981) were assayed for Fe by the total Fe procedure of Doeg & Ziegler (1962). The measured iron content of individual fractions was ≤0.25 mol/mol of enzyme subunit. Analysis of a pooled, concentrated enzyme preparation by the same procedure gave a value of 0.13 mol of Fe/mol of enzyme subunit. A value of 0.45 mol of Fe/mol was obtained after wet-ashing (Beinert, 1978). Enzyme was prepared from bacteria grown in the presence of ^{55}Fe (see Experimental Section) to test whether the low levels of Fe observed were associated with the purified enzyme or were merely adventitious. Determination of the radioactivity present in individual hydroxylapatite column fractions across the enzyme peak (maximum NADH:ubiquinone oxidoreductase specific activity = 570 units mg^{-1}) indicated <0.02 mol of Fe/mol of subunit. Even allowing for the possibility of some exchange with adventitious unlabeled Fe during column chromatography, it would seem that the solubilized enzyme does not possess an iron-containing prosthetic group. The absence of stoichiometric heme or iron–sulfur prosthetic groups in the purified enzyme can also be inferred from the visible and near-ultraviolet spectrum (see above). Sulfide analysis of the purified enzyme by the methylene blue method revealed <0.1 mol of acid-labile sulfide/mol of subunit, providing further confirmation of the absence of iron–sulfur clusters in the purified enzyme.

Other Metals. Cu, Mo, and W analyses on pooled enzyme from hydroxylapatite chromatography all gave negative results (Cu <0.02 and Mo and W <0.01 mol/mol of subunit) .

Phosphate Determination. Phosphate determinations on the enzyme polypeptide obtained by gel filtration of the purified enzyme in the presence of $NaDodSO_4$ (see Experimental Procedures) showed <0.1 mol of P/mol of subunit. This demonstrates that chromatography under these conditions results in complete removal of phospholipid from the preparation and also indicates that the enzyme does not contain any form of covalently bound P. This is of interest in view of the recent suggestion that the presence of covalently bound phosphate in flavoproteins may be a general occurrence (Edmondson & James, 1979).

Table I: Amino Acid Composition of NADH Dehydrogenase[a]

amino acid	nondelipidated[b]	delipidated[c]	sequence[d]
Asx	41.5	40.6	41
Thr	25.4	27.0	26
Ser	17.6	19.2	20
Glx	42.5	41.7	37
Pro	17.2	17.7	17
Gly	40.9	38.3	42
Ala	40.4	39.6	39
Val	28.1	29.7	27
Met	12.5[e]	12.5[e]	14
Ile	27.3	27.1	26
Leu	48.1	47.2	51
Tyr	9.1[f]	6.5[f]	12
Phe	11.9	11.2	11
His	15.5	15.0	17
Lys	87.4	25.8	25
Arg	21.9	21.6	22
Cys	3.9[g]	3.9[g]	4
Trp	2.6[h]	2.6[h]	2

[a] For details see the text. [b] Data averaged from duplicate 22-, 48-, and 96-h hydrolyses. Ser and Thr values were obtained by extrapolation to 0 h and Val and Ile values from the results of 48- and 96-h hydrolyses. [c] Data averaged from duplicate 22-h hydrolyses. Ser, Thr, Val, and Ile values were corrected by using appropriate destruction and slow release factors derived from the nondelipidated analyses. [d] Derived from the DNA sequence of the *ndh* gene (Young et al., 1981). [e] Determined separately as methionine sulfone on performic acid oxidized, delipidated samples hydrolyzed for 22 h. [f] Low values attributed to oxidative losses under the hydrolytic conditions employed. [g] Determined separately as cysteic acid on performic acid oxidized, delipidated samples hydrolyzed for 22 h and corrected by assuming 94% recovery (Moore, 1963). [h] Determined separately by mercaptoethanesulfonic acid hydrolysis of delipidated samples (Penke et al., 1974).

Amino Acid Composition. The amino acid composition of the respiratory NADH dehydrogenase and of solvent-extracted preparations is shown in Table I. In general, estimates for amino acids were not affected by delipidation, and there is good agreement with the amino acid composition predicted by the gene sequence (Young et al., 1981). However, as shown in Table I, the high lipid content of the preparation interferes with the lysine estimation, presumably because of the presence of ninhydrin-positive compounds derived from phosphatidylethanolamine.

Peptide Mapping. Two-dimensional tryptic peptide maps of the respiratory NADH dehydrogenase showed a total of 39 spots when stained with ninhydrin. Amino acid analysis of the insoluble "core" material indicated that less than 20% of the digest was relatively insoluble and that this might include a further seven potential peptides. Consideration of the predicted amino acid sequence (Young et al., 1981; Poulis et al., 1981) indicates that 39 tryptic peptides plus free arginine and lysine would be expected. Therefore, the number of peptides observed by mapping agrees with the predicted value within the experimental limits of the method. This demonstrates that the enzyme preparation does not contain two or more dissimilar polypeptides of identical molecular weight.

Delipidated enzyme was cleaved with CNBr, and the more soluble peptides were resolved by paper chromatography and electrophoresis. The amino acid compositions of two of the CNBr peptides are shown in Table II. One of these was positively identified as the carboxyl-terminal peptide because it contains no homoserine, and knowledge of its amino acid

Table II: Amino Acid Composition of N- and C-Terminal CNBr Peptides[a]

amino acid	N-terminal peptide	C-terminal peptide
Lys	1.8 (2)	1.0 (1)
His		1.0 (1)
Arg		3.1 (3)
Asx		
Thr	2.0 (2)	
Ser		1.2 (1)
Glx	1.4 (1)	
Pro	0.9 (1)	1.0 (1)
Gly	5.4 (5)	1.4 (1)
Ala	1.2 (1)	
Val	1.9 (2)	1.8 (2)
Ile	1.6 (2)	1.7 (2)
Leu	2.3 (2)	3.1 (3)
Hse[b]	0.6 (1)	0.0 (0)
amt recovd (nmol/residue)	9	9

[a] CNBr peptides were produced, purified, and analyzed as described under Experimental Procedures. Values shown are normalized to give the best fit to predicted compositions. Analytical values < 3 nmol (except Hse) are not shown. Values in parentheses are the integral number of each residue predicted from the DNA sequence (Young et al., 1981). [b] Homoserine plus homoserine lactone.

composition was useful in identifying and confirming the carboxyl-terminal portion of the structural gene in the DNA sequence (Young et al., 1981). The amino-terminal CNBr peptide is derived from the amino-terminal 19 residues of the mature, processed enzyme (Young et al., 1981; Poulis et al., 1981).

Reconstitution of NADH Oxidase in ndh Mutant Membrane Particles. Experiments were undertaken to see whether an NADH oxidase activity could be reconstituted from purified NADH dehydrogenase and membrane particles prepared from *ndh* mutant strains, which lack a functional respiratory NADH dehydrogenase (Young & Wallace, 1976; Jaworowski et al., 1981). Varying amounts of purified enzyme were added to a fixed volume of IY12 membrane particles at 4 °C, and the resulting mixture was assayed for NADH oxidase activity. The rates of NADH oxidation obtained were corrected for both the low residual NADH oxidase activity present in the mutant membrane particles (1–2% of the wild-type levels) and the activity of the purified enzyme, which exhibits an NADH dehydrogenase activity with O_2 as the electron acceptor ~1% of that with ubiquinone-1, under the assay conditions used.

As can be seen in Figure 3a, the addition of purified NADH dehydrogenase to IY12 membrane particles reconstituted NADH oxidase activity. This activity, in contrast to those present prior to reconstitution, is sensitive to KCN (see below). Under the conditions of the above experiment, a linear relationship was observed between the amount of added enzyme and reconstituted NADH oxidase activity.

It was observed that the purified enzyme preparation is no longer reconstitutively active after dialysis against low ionic strength buffer (Figure 3b). This phenomenon was of interest since it appeared possible that some component of the enzyme necessary for electron transfer to endogenous ubiquinone-8, but not for ubiquinone-1 reduction, was being lost during dialysis. However, the loss of the ability to reconstitute *ndh* mutant membrane particles after dialysis against low ionic strength buffer was found to be reversible, since a reconstitutively active preparation could be obtained by further dialysis of such reconstitutively inactive preparations against 1 M phosphate buffer (Figure 3b). This did not simply reflect a phosphate ion dependence in the assay, however, since addition of equivalent amounts of phosphate buffer to the assay did not result in reconstitution by the inactive preparations. It is therefore unlikely that a loss of any component of the enzyme during dialysis leads to a reconstitutively inactive preparation, and a change in the state of aggregation or a conformational change is probably responsible.

For determination of the distribution of the reconstituted NADH oxidase activity, reconstituted particles were diluted and membranes were collected by centrifugation. The particulate fraction contained 100% of the cyanide-sensitive NADH oxidase and 98% of the total NADH oxidase activity (Table III). Comparison of the NADH:ubiquinone oxidoreductase activity of the particulate and soluble fractions indicated that 53% of the added enzyme had become associated with the membrane fraction and was catalyzing the transfer of electrons from NADH to the respiratory chain, presumably at the level of the endogenous ubiquinone-8.

From the results presented in Table III, the efficiency of reconstitution (defined as units of reconstituted NADH oxidase activity per unit of added NADH:ubiquinone oxidoreductase activity) was estimated to be 38%. This is a direct measure of the proportion of added enzyme interacting with the respiratory chain if the respective specific activities of the pure enzyme and the membrane-bound enzyme are identical. In wild-type membrane particles, where the respiratory NADH dehydrogenase is rate limiting in the overall NADH oxidase reaction (Young et al., 1978), the rate of NADH-dependent ubiquinone-1 reduction is similar to that of the NADH oxidase reaction. This indicates that for the enzyme in vivo the rate of reaction with saturating concentrations of ubiquinone-1 as the electron acceptor is not substantially different to that with the endogenous ubiquinone-8. It can also be calculated from the results in Table III that, subject to the above assumption, essentially all of the added enzyme which becomes membrane-associated interacts with the respiratory chain to reconstitute NADH oxidase activity.

Table III: Distribution of Reconstituted NADH Oxidase Activity[a]

	prior to reconstitution			after centrifugation	
activity	IY12 membrane particles	purified enzyme	reconstituted particles	particle fraction	supernatant
NADH oxidase	0.115	0.232	5.78	6.84	0.154
NADH oxidase[b] (plus KCN)	0.062	0.274	0.821	0.412	0.146
NADH:ubiquinone oxidoreductase	0.426	16.6	14.9	5.60	4.64

[a] IY12 membrane particles (500 μL; 30 mg of protein mL^{-1}) were mixed with 250 μL of purified NADH:ubiquinone oxidoreductase (73 units mL^{-1}) at 4 °C. The reconstituted particles were diluted with 3.25 mL of ice-cold STM buffer [0.1 M Tes, pH 7.5, containing 0.25 M sucrose and 0.02 M $Mg(CH_3COO)_2$] and centrifuged for 3 h at 47 000 rpm at 4 °C in a Beckman SW56 rotor. The pellet was resuspended to 2 mL in STM buffer. As a control, 250 μL of enzyme was mixed with 3.75 mL of STM buffer and centrifuged as above. Under these conditions, in the absence of membrane particles, 1.12 units of NADH:ubiquinone oxidoreductase pelleted. The overall recovery of NADH:ubiquinone oxidoreductase activity in the control after centrifugation was identical with that obtained above (60%). [b] Assayed in the presence of 3 mM KCN.

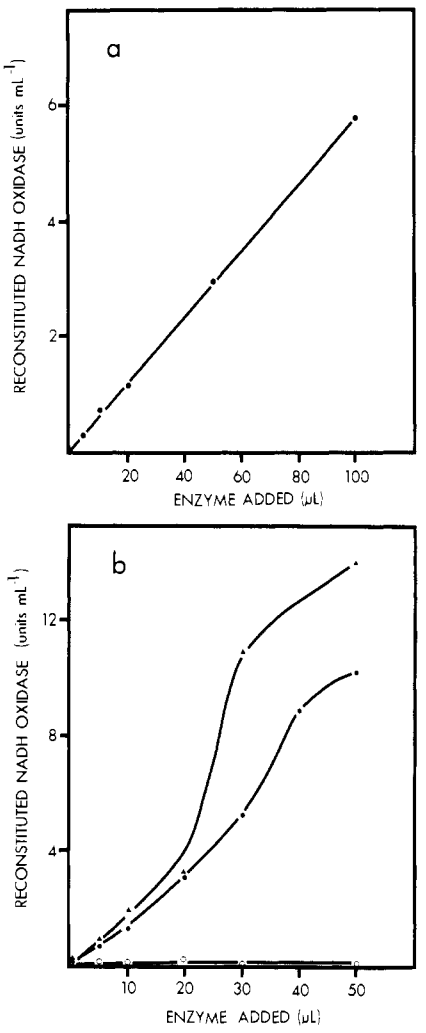

FIGURE 3: Reconstitution of NADH oxidase in *ndh* mutant membrane particles. (a) Reconstitution of NADH oxidase activity in IY12 membrane particles by method 1 (see Experimental Procedures for details). Various volumes of purified enzyme were added to 50-μL membrane particles, which were then assayed for NADH oxidase activity (●). (b) Reversible loss of reconstitutive activity at low ionic strength. 2 × 1 mL aliquots of purified NADH:ubiquinone oxidoreductase (19.4 units mL^{-1} in ~0.7 M potassium phosphate buffer, pH 7.5, containing 0.1% cholate and 20 μM FAD) were dialyzed for 3 h at 4 °C against 100 mL of 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% cholate. One sample was then further dialyzed for 3 h at 4 °C against 100 mL of 1 M potassium phosphate buffer, pH 7.5, containing 0.1% cholate. Aliquots (5 μL) of IY12 membrane particles were treated with various volumes of the two dialyzed preparations and an undialyzed control preparation of NADH:ubiquinone oxidoreductase by method 2 (see Experimental Procedures for details), prior to measurement of NADH oxidase activity. (▲) Undialyzed control; (○) dialyzed vs. 5 mM buffer; (●) redialyzed vs. 1 M buffer.

The ability of the purified enzyme to reconstitute other *ndh* mutants apart from IY12 was tested. The purified NADH dehydrogenase was shown to reconstitute NADH oxidase activity in membrane particles derived from each of the twelve *ndh* mutant strains isolated so far (Young & Wallace, 1976), indicating that they are all defective in the primary dehydrogenase.

Discussion

In previous studies we have purified the respiratory NADH dehydrogenase of *E. coli* from membrane preparations of genetically amplified strains (Young et al., 1978; Jaworowski et al., 1981). The purified enzyme shows a single band of apparent molecular weight 45 000 on $NaDodSO_4$ gel electrophoresis. The amino acid sequence of the enzyme has been determined via the nucleotide sequence of the cloned *ndh* gene (Young et al., 1981) and gives a molecular weight of 47 200 for the enzyme polypeptide.

In the present work the purified enzyme has been characterized in detail. The amino acid composition before and after solvent delipidation is in good agreement with the composition predicted from the gene sequence, and the predicted amino- and carboxyl-terminal CNBr peptides have been isolated. Thus, apart from posttranslational cleavage of the amino-terminal *N*-formylmethionine residue (Poulis et al., 1981), the mature enzyme corresponds to the entire coding sequence of the *ndh* gene.

The enzyme contains 1 mol of noncovalently bound flavin/mol of subunit. Chromatography of flavin extracts, coupled with reactivation studies on flavin-depleted preparations, has shown that the enzyme is FAD specific. There is no covalently bound flavin present or any other covalently bound prosthetic groups which contain phosphate or possess detectable visible or ultraviolet absorption. A notable feature of the preparation is its very high lipid content. This is presumably due to the use of cholate in solubilizing the enzyme, since, in general, this detergent does not totally disrupt protein–lipid interactions (Helenius et al., 1979). It is not clear at present how much lipid is tightly associated with the enzyme, and the question of whether lipid is required for catalytic activity is currently under investigation.

The purified enzyme contains only low levels of Fe and acid-labile S. While it is possible that an iron–sulfur cluster could have been lost during purification, it is clear that iron is not involved in the efficient ubiquinone-1 reduction catalyzed by the enzyme in vitro. The *E. coli* NADH dehydrogenase has the highest NADH:ubiquinone oxidoreductase specific activity (500–600 μmol min^{-1} mg^{-1} at pH 7.5, 30 °C) of any enzyme reported so far and the apparent K_m for ubiquinone-1 (<5 μm) is low (Jaworowski et al., 1981). These observations suggest that ubiquinone-8 may be the immediate acceptor for the enzyme in vivo. Work is in progress to test this more directly, but in this connection it is interesting to note that the purified enzyme contains near-stoichiometric amounts of ubiquinone-8.

The ability of the purified enzyme to efficiently reconstitute cyanide-sensitive NADH oxidase activity in membrane particles prepared from *ndh* mutant strains indicates that the activity of the enzyme with its immediate physiological electron acceptor has been preserved. The enzyme appears able to integrate into the membrane and interact normally with the other components of the respiratory chain. This suggests that in vivo, the enzyme could insert into the membrane after synthesis and folding are completed, rather than commencing insertion as a nascent, ribosome-bound polypeptide. This would be consistent with the results of DNA and protein sequencing, which show that a cleavable signal peptide (Davis & Tai, 1980) is not involved (Young et al., 1981; Poulis et al., 1981).

Added in Proof

Recent experiments have further confirmed that the reduction of ubiquinone-1 by the purified enzyme does not involve iron. After gel filtration in the presence of EDTA and 2-mercaptoethanol, we have obtained a preparation with an NADH:ubiquinone-1 oxidoreductase specific activity of ~900 units/mg of protein, and a total iron content of <0.1 mol of Fe/mol of protein, measured colorimetrically after wet-ashing (H. D. Campbell, unpublished data).

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