Stereospecificity and Requirements for Activity of the Respiratory NADH Dehydrogenase of *Escherichia coli*†

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ABSTRACT: The respiratory NADH dehydrogenase of *Escherichia coli* has been further amplified in vivo by genetic methods. The enzyme, a single polypeptide of M_r 47 200 of known amino acid sequence [Young, I. G., Rogers, B. L., Campbell, H. D., Jaworowski, A., & Shaw, D. C. (1981) *Eur. J. Biochem. 116*, 165–170], constitutes 10–15% of the total protein in the amplified membranes. In situ in the membrane, the enzyme contains 1 mol of FAD/mol of subunit and has a specific NADH:ubiquinone-1 oxidoreductase activity of ~1100–1200 units mg^{-1} at 30 °C, pH 7.5. The purified enzyme contains phospholipid, which remains closely associated with it during gel filtration on Sephacryl S-300 in the presence of 0.1% (w/v) cholate at low ionic strength. Under these conditions the enzyme is extensively aggregated (apparent M_r $>10^6$). This procedure yielded enzyme with a specific activity of 980 units mg^{-1}, similar to the value observed in the membrane. This preparation contained <0.1 mol of Fe/mol of enzyme, confirming that Fe is not involved in reduction of ubiquinone 1 catalyzed by the enzyme. Neutron activation analysis of purified enzyme has demonstrated the absence of 35 trace elements including Se, Zn, Mn, Co, W, Cu, and Fe. The enzyme polypeptide, prepared completely free of phospholipid, FAD, and ubiquinone by gel filtration in the presence of sodium dodecyl sulfate, has been reactivated. The results show that the only components necessary for catalysis of ubiquinone-1 reduction by NADH in this system are the enzyme polypeptide, FAD, and phospholipid. The stereospecificity of hydride transfer from the dihydronicotinamide ring of NADH catalyzed by the enzyme both in purified form and in wild-type membranes has been shown to be 4-*pro-S* ("B"). This is the same as that of the mitochondrial respiratory NADH dehydrogenase and of a group of reduced pyridine nucleotide:disulfide oxidoreductase flavoproteins which includes glutathione reductase. The stereospecificity is different from that of a number of other activities involved in electron transport processes or quinone reduction, which were under consideration as being evolutionarily related (e.g., NADH: cytochrome b_5 reductase).

The respiratory NADH dehydrogenase of *E. coli* is the first component of the membrane-bound energy-conserving NADH oxidase system, which is located in the inner, or cytoplasmic, membrane.

The structural gene (*ndh*)[1] coding for the respiratory NADH dehydrogenase has been cloned (Young et al., 1978). The DNA sequence of the gene, in combination with results of protein chemical studies, has given the complete amino acid sequence of the protein (Young et al., 1981; Jaworowski et al., 1981b; Poulis et al., 1981). The mature enzyme consists of a single polypeptide chain of 433 residues with M_r 47 200. The only processing of the protein detected is the removal of the initiating *N*-formylmethionine residue from the N-terminus (Young et al., 1981; Poulis et al., 1981).

The construction of strains that overproduce the enzyme has enabled the large-scale isolation of this membrane-bound protein. The enzyme is purified in a single step by chromatography of solubilized membranes on hydroxylapatite.

The purified enzyme retains high catalytic activity toward ubiquinone as acceptor, and previous studies have shown that it contains phospholipid, FAD (1 mol/mol of protein), and ubiquinone 8 (~1 mol/mol of protein), in addition to the enzyme polypeptide of M_r 47 200 (Jaworowski et al., 1981b). In the present work we have investigated the requirements for enzyme activity and have established the stereospecificity of hydride transfer.

Experimental Procedures

Materials. The sources of many chemicals used have been described previously (Jaworowski et al., 1981a). 2-Mercaptoethanol was from Eastman. Urea (Ultrapure) was from BRL. AG1-X2 and Chelex-100 were from Bio-Rad. $NaDodSO_4$ ("specially pure"), acetaldehyde, and TMAB were from BDH. Lipoamide, pig heart lipoamide dehydrogenase, DTT, and yeast alcohol dehydrogenase were the best grades available from Sigma. ^2H_2O was a gift from Dr. R. I. Christopherson. Crude soybean L-α-phosphatidylcholine was from Sigma (type II-s) and was purified as described by Kagawa & Racker (1971).

Bacterial Strains and Plasmids. Strains IY91, IY92, and IY93 are derivatives of the *E. coli* K12 strains IY12 (*thi, his, ilv, trp, rpsL, ndh*) or its isogenic *ndh*$^+$ transductant IY13 (Young & Wallace, 1976). Strain IY91 has been described previously (Jaworowski et al., 1981b). It carries plasmid pIY10, constructed by ligating the *ndh* gene (Young et al., 1978) and a double *lac* promoter fragment (Johnsrud, 1978) into the *Eco*RI site of pMB9 (Rodriguez et al., 1976). Strains IY92 and IY93 were constructed by transformation of IY12 and IY13 respectively with pLJ3 (Johnsrud, 1978), which carries a double *lac* promoter fragment inserted at the *Eco*RI site of pMB9. Transformation was accomplished as described previously (Poulis et al., 1981), and transformants were selected for resistance to tetracycline. Viable colonies were tested for the presence of multiple copies of the *lac* promoter carried by pLJ3 by using indicator plates containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Johnsrud, 1978). The *ndh*$^-$ and *ndh*$^+$ phenotypes of IY92 and IY93, respectively, were verified by examination of their growth characteristics on mannitol-minimal plates

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[1] Abbreviations: *ndh*, structural gene for NADH dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; ubiquinone *n*, ubiquinone isoprenologue containing *n* isoprene units in the side chain; EDTA, ethylenediaminetetraacetic acid disodium salt; DTT, dithiothreitol; TMAB, tetramethylammonium bromide; Cl_3CCOOH, trichloroacetic acid; BSA, bovine serum albumin; NMR, nuclear magnetic resonance; SD, standard deviation; Tris, tris(hydroxymethyl)aminomethane.

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(Young & Wallace, 1976). Finally, examination of the NADH oxidase and NADH:ubiquinone-1 oxidoreductase levels of membranes prepared from IY92 and IY93 showed that these levels were similar to those of the parent strains IY12 and IY13.

Preparation of Membranes. Strain IY91 was grown, and membranes were prepared as described previously (Jaworowski et al., 1981a,b), except that in some cases, enzyme levels were enhanced by chloramphenicol amplification of plasmid copy number (Jaworowski et al., 1981a; Young et al., 1978). Strains IY92 and IY93 were grown in similar fashion, and membranes were prepared after chloramphenicol treatment as described.

Preparation of Respiratory NADH Dehydrogenase. Enzyme was prepared and concentrated as described previously (Jaworowski et al., 1981a,b).

Determination of Flavin. The flavin levels of amplified membranes were determined as follows. To 100 μL of membrane preparation in STM buffer (0.25 M sucrose, 0.1 M Tes, and 0.02 M magnesium acetate, pH 7.5) were added 1.0 mL of H_2O and 100 μL of 72% (w/v) Cl_3CCOOH. After vortexing, the mixture was spun for 10 min at 12000*g* in an Eppendorf Model 5412 centrifuge at 4 °C. The absorption spectrum from 550 to 320 nm of the supernatant was then recorded. The blank for the base-line spectrum was prepared as above except that 100 μL of STM buffer or H_2O replaced the membranes. The concentration of flavin was determined from A_{445} values by using $E_{M,445nm}$ = 10 200 M^{-1} cm^{-1}, determined under the conditions of the assay. Solutions of FAD were standardized spectrophotometrically by using $E_{M,450nm}$ = 11 300 M^{-1} cm^{-1} (Whitby, 1953). Although there are slight differences between the absorption spectra of FAD and FMN at neutral pH in the region examined, in 6% (w/v) Cl_3CCOOH, the spectra are identical.

Flavin values were corrected for the slight (~10%) background absorbance in the assay when membranes from any of the strains IY12, IY13, IY92, or IY93 were substituted in the assay.

Flavin was isolated from chloramphenicol-amplified IY91 membranes by heat denaturation and phenol extraction as described by Fazekas & Kokai (1971). The flavin content of these extracts was examined by thin-layer chromatography on silica gel (Merck, F_{254}, 0.25 mm) in solvent systems I and IV of Fazekas & Kokai (1971).

Borohydride Reduction. The borohydride reducibility (Crane & Barr, 1971) of fractions from Sephacryl S-300 chromatography in the presence of 1% (w/v) $NaDodSO_4$ and 0.05 M NH_4HCO_3 was tested before and after addition of 300 μL of ethanol to 1 mL of the column fraction. Control experiments showed that this amount of ethanol greatly facilitated reduction of exogenous ubiquinone 8, added as an ethanolic solution to 1% (w/v) $NaDodSO_4$ and 0.05 M NH_4HCO_3. The enzyme polypeptide precipitated at higher concentrations of ethanol.

$NaDodSO_4$ Gel Electrophoresis. Electrophoresis on 10% and 15% $NaDodSO_4$ slab gels, staining with Coomassie Blue R250, and estimation of enzyme levels by densitometry of stained gels were performed as described previously (Jaworowski et al., 1981a; Young et al., 1978). A correction was applied for the staining present in the M_r 47 000 region on control gels run on known amounts of IY92 and IY93 membrane protein.

Determination of Protein. Protein was determined by the method of Lowry et al. (1951) with BSA as standard. Stock solutions of BSA were standardized spectrophotometrically by using $A^{1\%}_{1cm,280nm}$ = 6.7.

In order to facilitate calculation of Lowry protein assay data, we sought a mathematical transformation that would linearize the standard curve. We tested the log absorbance vs. log [protein] plot proposed by Bates & McAllister (1974), Stauffer (1975), and Peterson (1977) but found that it yielded a curve. Coakley & James (1978) showed that data for bovine serum albumin were linearized by use of a double-reciprocal plot. We have tested the double-reciprocal plot (1/absorbance vs. 1/[protein]) on several proteins with a wide variation in tryptophan and tyrosine constant (bovine serum albumin, bovine pancreatic ribonuclease A, and chicken egg white lysozyme). All three show excellent linearity in the double-reciprocal plots up to very high protein concentrations (final concentration in assay 1500 μg mL^{-1}). The data fit the Michaelis–Menten-type equation $A = A_{max}/(1 + k/[protein])$, where A is the absorbance corresponding to any particular [protein], A_{max} is the theoretical absorbance at infinite protein concentration (and is essentially the same for different proteins), and k is a constant which has a particular value for each protein. From this, it is readily shown that under conditions where two proteins both show the same absorbance in the assay, [protein 1] = (k_1/k_2)[protein 2] where k_1 and k_2 are the constants described above for proteins 1 and 2, respectively. This shows that, in spite of the nonlinearity of the assay data and the different amounts of color generated by different proteins (Lowry et al., 1951), the linear transformation described provides a valid estimate of protein concentration.

Lowry assay results for purified *E. coli* respiratory NADH dehydrogenase were converted to absolute protein concentrations by using the factor k_1/k_2 of 0.88 determined by amino acid analysis as described previously (Jaworowski et al., 1981b).

Other Analytical Methods. NADH:ubiquinone oxidoreductase and NADH oxidase activities were determined spectrophotometrically (Jaworowski et al., 1981a). Phospholipid was determined by phosphate analysis after wet-ashing (Jaworowski et al., 1981b), and iron was determined by the method of Beinert (1978).

Purification of Enzyme Polypeptide by Gel Filtration in $NaDodSO_4$. The enzyme polypeptide was stripped of lipid and flavin by chromatography on a 65 × 2.6 cm column of Sephacryl S-300 equilibrated with 0.05 M NH_4HCO_3 and 1% (w/v) $NaDodSO_4$, as described previously (Poulis et al., 1981). Fraction volumes were measured by weight. Protein was determined by measuring A_{280} or by Lowry determinations. The integrity of the polypeptide chain was routinely monitored by $NaDodSO_4$ gel electrophoresis. Peak tubes were pooled and used for reconstitution (see later). Standard proteins (ovalbumin, BSA, and myoglobin) were chromatographed after treatment with $NaDodSO_4$ under similar conditions.

Reconstitution of Enzyme Activity from NADH Dehydrogenase Polypeptide after Gel Filtration in $NaDodSO_4$. Removal of $NaDodSO_4$ from the protein by Dowex 1-X2 in the presence of 6 M urea was accomplished essentially according to Weber & Kuter (1971). The NADH dehydrogenase polypeptide in $NaDodSO_4$ solution was used for reconstitution immediately after preparation. In a typical experiment, 10.0 mL of the polypeptide (0.24 mg/mL) in 0.05 M NH_4HCO_3 and 1% (w/v) $NaDodSO_4$ was treated with 7 μL of 2-mercaptoethanol and 3.6 g of urea with magnetic stirring. After the urea had dissolved, the solution was incubated at room temperature for 30 min. Then 2 mL of a suspension of AG1-X2 (corresponding to 1 mL packed resin), preequilibratd with 0.05 M Tris/acetate, pH 7.8, containing 0.01 M 2-mercaptoethanol and 6 M urea, was added, and the

solution was stirred at room temperature for a further 30 min to remove $NaDodSO_4$. The mixture was then filtered through a 0.4-μm Millipore filter and used immediately for renaturation. Renaturation was accomplished by a 20-fold dilution of the solution at 4 °C into a variety of buffers containing various additives (see Results for details). Enzyme activity was determined after overnight incubation at 4 °C.

Neutron Activation Analysis. Concentrated enzyme from hydroxylapatite chromatography (specific activity 530 units mg^{-1}; stored at −60 °C until use) was dialyzed vs. 2 × 400 volumes of 5 mM Tes buffer, pH 7.5, containing 0.1% potassium cholate and 20 μM FAD, at 0–4 °C. After dialysis the enzyme had a protein concentration of 3.4 mg mL^{-1} and an NADH:ubiquinone oxidoreductase specific activity of 169 units/mg of protein. An amount of the enzyme containing 17.8 mg of protein and 22.9 mg of phospholipid (88 mol of phospholipid/mol of enzyme) together with lower amounts of Tes, cholate, and FAD was lyophilized and then kept on dry ice until subjected to instrumental neutron activation analysis at the Australian Atomic Energy Commission reactor at Lucas Heights, Sydney, under the direction of Dr. T. M. Florence.

Determination of Stereospecificity. (4*S*)-[4-^{2}H]NADH was prepared by reduction of NAD^+ catalyzed by pig heart lipoamide dehydrogenase as described previously (Arnold & You, 1978). Complete oxidation of (4*S*)-[4-^{2}H]NADH catalyzed by purified NADH dehydrogenase with ubiquinone 1 as acceptor was achieved as follows. To 20–30 mL of H_2O containing 5 mM potassium phosphate buffer, pH 7.5, was added 10.7 mg of (4*S*)-[4-^{2}H]NADH. To the magnetically stirred solution at room temperature was added 1 mL of 16.4 mM ubiquinone 1 in ethanol. Enzyme from chromatography on Sephacryl S-300 in the presence of cholate (2 μL; 18 units of NADH:ubiquinone-1 oxidoreductase activity, specific activity 870 units/mg of protein) was added. The A_{340} of the solution was monitored after dilution of 100-μL aliquots into 1 mL of H_2O in a spectrophotometer cell.

In one experiment, the reaction was run in 20 mL of ^2H_2O, and further enzyme (total 9 μL) was added to speed up the reaction. When the wild-type membrane NADH oxidase was studied, the reaction was carried out at 37 °C, ubiquinone 1 was omitted, and 29 μL (1 mg of protein) of washed IY13 membranes was added instead of purified enzyme. Oxidation of the (4*S*)-[4-^{2}H]NADH catalyzed by alcohol dehydrogenase was carried out with a reaction mixture consisting of 30 mL of H_2O containing 5 mM potassium phosphate buffer, pH 7.5, 10.9 mg of (4*S*)-[4-^{2}H]NADH, 20 μmol of acetaldehyde, and 0.12 mg of yeast alcohol dehydrogenase.

When reactions were complete, the pH (or pD) was adjusted to 2.0 with HNO_3, and when ubiquinone 1 was present, the solution was then extracted twice with 10 mL of diethyl ether (total 20 mL) to remove ubiquinone and ubiquinol. Ether was removed from the water layer with a stream of high purity N_2 at 37 °C. The acidified solution was filtered through a 0.4-μm Millipore filter and lyophilized. When membranes were used, the acidified solution was centrifuged 10 min at 1750*g* before filtration. In some instances it was found necessary to eliminate NMR peak broadening caused by paramagnetic metal ion impurities by passage of the product NAD^+ dissolved in ~1 mL of ^2H_2O through a column of 0.50 g wet weight of Chelex-10 (200-400 mesh, Na^+ form). The NAD^+ solution was then lyophilized.

For NMR spectroscopy, the NAD^+ was dissolved in ~0.6 mL of ^2H_2O containing 1 mM EDTA and 1 mM TMAB, pD ~3. NMR spectra were recorded at 30 °C on a JEOL FX-90Q spectrometer operated in the Fourier transform mode. Typically, 100–200 scans, each consisting of 16000 data points, were accumulated for each spectrum.

Integration of the dihydropyridine C4-H_2 resonance (Meyer et al., 1962) in NMR spectra of the (4*S*)-[4-^{2}H]NADH gave a value of 1.41 protons, showing that the sample contained 41% ^{1}H and 59% ^{2}H at the 4*S* position. Results were corrected by using these values. Authentic NADH (P-L, "Chromatopure") gave a value of 2.04 protons for the C4-H_2 peak. The presence of deuterium exclusively at the 4*S* position is established by the stereospecificity of the enzymatic synthesis, as well as by the results of the present experiments (see Results).

The lack of complete deuteration of the 4*S* position of the NADH may be due to the presence of ^{1}H (≈3%, estimated by NMR spectroscopy) in the ^2H_2O used in the synthesis (k_H/k_D for lipoamide dehydrogenase is unreported) or to exchange with ^1H_2O during the preparation. With regard to the latter, after reduction of the NAD^+ in ^2H_2O catalyzed by lipoamide dehydrogenase, the reaction mixture was diluted with ^1H_2O prior to DEAE-cellulose chromatography. Dilution with ^2H_2O would eliminate the possibility of exchange catalyzed by lipoamide dehydrogenase.

Results

Elevated Level of FAD in IY91 Membranes. Membrane vesicle preparations from strain IY91, which contains a multicopy plasmid consisting of the *ndh* gene, a *lac* promoter fragment, and pMB9 as vector (Jaworowski et al., 1981b), were appreciably more yellow in color than membranes from appropriate control strains (IY92 and IY93). Very high levels of flavin, identified as FAD, were present in these membrane preparations. The amount of enzyme protein present showed a good correlation with the amount of flavin present in the membrane preparations. For a series of IY91 membranes which varied from 6 to 13% NADH dehydrogenase (percentage of total protein), the ratio of FAD to enzyme protein was measured as 1.06 ± 0.14 (SD) mol/mol (n = 6). The measured NADH:ubiquinone-1 activity of the membrane preparations allowed the calculation of the specific NADH:ubiquinone-1 activity of the enzyme in situ in these highly amplified membranes as 1110 ± 210 (SD) units mg^{-1}, based on flavin, or 1170 ± 290 (SD) units mg^{-1}, based on gel densitometry (n = 6).

Gel Filtration of Purified Enzyme at Low Ionic Strength. Preparations of the respiratory NADH dehydrogenase from hydroxylapatite chromatography of solubilized membranes in the presence of 0.1% cholate contain large amounts of lipid, predominantly phosphatidylethanolamine (Jaworowski et al., 1981b). In order to examine the association of lipid and protein, and to obtain information concerning the aggregation state of the enzyme, such a preparation was subjected to gel filtration on Sephacryl S-300 equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.1% cholate, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 20 μM FAD.

The enzyme eluted as a single peak near the void volume of the column (apparent $M_r > 10^6$). There was some tailing of the peak to lower apparent molecular weight values. The activity, protein, and phospholipid peaks were all more or less coincident. The phospholipid to protein ratio decreased somewhat across the peak. This ratio had a value of 53 mol of phospholipid/mol of protein at the top of the peak, identical with that of the applied enzyme. The procedure brought about an increase in the specific activity of the enzyme to a constant value of 980 units mg^{-1} across the peak. This value is similar to the specific NADH:ubiquinone-1 activity of the enzyme in situ in the membrane (see above) and is the highest specific

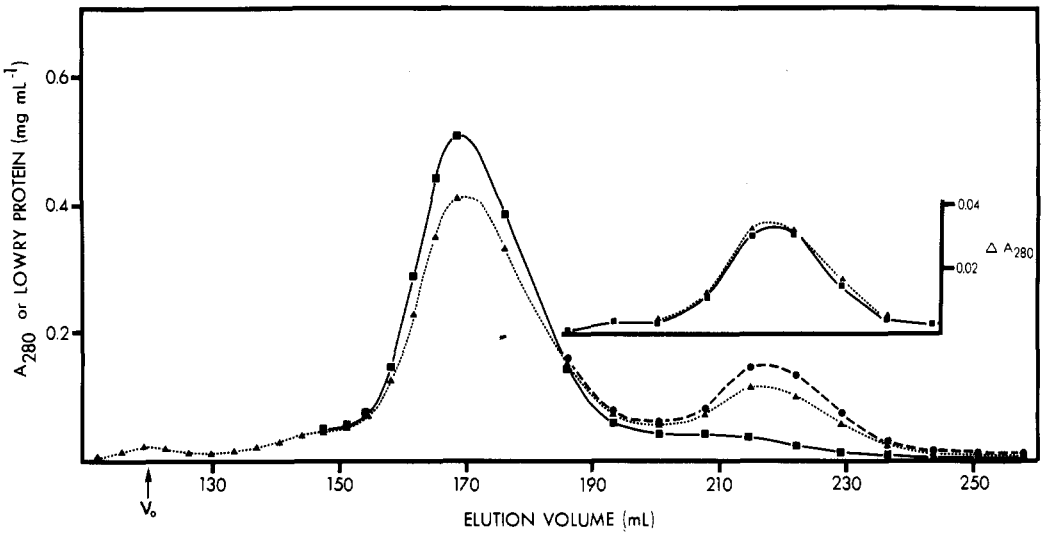

FIGURE 1: Separation of NADH dehydrogenase polypeptide from ubiquinone 8 by chromatography on Sephacryl S-300 in the presence of $NaDodSO_4$. Purified NADH dehydrogenase was chromatographed on a 65 × 2.6 cm column of Sephacryl S-300 equilibrated with 0.05 M NH_4HCO_3 and 1% (w/v) $NaDodSO_4$, as described previously (Poulis et al., 1981). Lowry protein (■); A_{280} (▲); A_{280} after air oxidation (●). Inset: ΔA_{280} on air oxidation (■) or subsequent borohydride reduction (▲).

activity so far reported for any ubiquinone reductase.

After concentration to 10 mg of protein mL^{-1} by ultrafiltration, the pooled enzyme from S-300 chromatography had a specific activity of 870 units mg^{-1}, and on storage at 0–4 °C, protected from light, it lost activity with $t_{1/2} \sim 11$ weeks. This is the most stable, purified form of the enzyme we have obtained. The enzyme from hydroxylapatite chromatography (Jaworowski et al., 1981b) exhibits somewhat variable stability and activity.

The iron content of the concentrated enzyme from S-300 chromatography was measured colorimetrically after wet-ashing with H_2SO_4 and H_2O_2. Values of <0.1 mol of Fe/mol of enzyme were obtained. This confirms that Fe is not involved in ubiquinone-1 reduction by the enzyme, since the specific activity of this enzyme preparation is similar to the specific NADH:ubiquinone-1 oxidoreductase activity of the enzyme in situ in the membrane, as shown in this paper.

Neutron Activation Analysis. In order to further examine the possible involvement of trace elements in catalysis by the enzyme, a large sample of enzyme was subjected to instrumental neutron activation analysis.

The following 35 elements were present at less than 0.1 mol/mol of enzyme (in some cases, trace amounts of the element were detected, and in others the element was not detected): Sc, V, Cr, Mn, Fe, Co, Cu, Zn, Ga, As, Se, Br, Sr, In, Sb, I, Cs, Ba, La, Ce, Nd, Sm, Eu, Tb, Dy, Er, Yb, Lu, Hf, Ta, W, Ir, Au, Th, U. Al was not detected at a detection limit of 0.22 mol/mol of enzyme. For some elements of interest, the actual levels detected or detection limits (in mol/mol of enzyme) were as follows: Fe, 0.082 ± 0.018; Cu, <0.081; Zn, 0.024 ± 0.002; Se, <0.0011; W, <0.0038. The absence of Mo has previously been demonstrated by chemical analysis (Jaworowski et al., 1981b).

Reactivation of Enzyme Polypeptide Isolated by Gel Filtration in $NaDodSO_4$. In order to investigate the requirements of the enzyme for activity, we decided to examine the reactivation of the enzyme polypeptide isolated by gel filtration in the presence of $NaDodSO_4$. We have previously shown that this procedure completely removes FAD and phospholipid from the polypeptide (Poulis et al., 1981; Jaworowski et al., 1981b).

In the present work, we have shown that endogenous ubiquinone 8 (Jaworowski et al., 1981b) is also separated from the polypeptide. Figure 1 shows a typical chromatogram. Calibration of the column with standard proteins showed that the NADH dehydrogenase polypeptide elutes with an apparent molecular weight of ~50000. The second major absorbance peak (ubiquinone 8) is essentially Lowry negative. The A_{280} of this peak increases on standing (Figure 1), suggesting air oxidation of the quinol form of Q-8 (resulting from reduction by 2-mercaptoethanol) back to the quinone. The oxidized form of the material in this peak shows $\lambda_{max} = 275$ nm and exhibits borohydride reducibility. The ΔA_{280} of individual fractions on air oxidation or subsequent borohydride reduction is similar (Figure 1, inset). The A_{280} of the protein is completely stable to air oxidation or borohydride reduction, demonstrating the absence of ubiquinone in the protein peak.

The enzyme polypeptide used for reconstitution was examined by $NaDodSO_4$ gel electrophoresis and exhibited good purity (Young et al., 1978; Jaworowski et al., 1981a). Furthermore, the presence of <0.1 mol of P/mol of enzyme after wet-ashing was demonstrated, confirming the absence of endogenous FAD and phospholipid.

The polypeptide was freed of $NaDodSO_4$ and reconstituted according to Weber & Kuter (1971) as described under Experimental Procedures. Some results are shown in Table I. The reconstitution shows a dependence on the presence of both FAD and phospholipid (Table I). Exogenous inert protein (BSA) enhanced the recovery of activity, as was observed in some cases previously (Weber & Kuter, 1971). The substrate NADH had little effect on the reconstitution (Table I). Although the maximum recovery of activity of 8% (Table I) may seem low, we regard this figure as satisfactory in view of the following: (i) the purified enzyme is rather unstable, (ii) soybean phospholipid was used, not *E. coli* phospholipid, (iii) no systematic attempt was made to optimize conditions for reconstitution, and (iv) recoveries with soluble proteins are often ≪100% (Weber & Kuter, 1971).

When these considerations are taken into account, the results provide good evidence that transfer of reducing equivalents from NADH to ubiquinone 1 catalyzed by the enzyme requires only the pure NADH dehydrogenase polypeptide, FAD, and phospholipid. There is no evidence of a role for any other cofactor or metal ion.

Reconstitution was also observed, with the same requirement for both FAD and phospholipid, with both 1 M potassium phosphate buffer, pH 7.5, and 0.1% cholate in 0.05 M

Table I: Reconstitution of NADH:Ubiquinone-1 Oxidoreductase Activity of Respiratory NADH Dehydrogenase Polypeptide following Gel Filtration in the Presence of $NaDodSO_4$[a]

additions				NADH: ubiquinone-1 oxidoreductase specific activity (units mg^{-1})[c]
phospholipid[b] (10 mg mL^{-1})	FAD (50 μM)	BSA (0.1 mg mL^{-1})	NADH (1 mM)	
−	−	−	−	<1
+	−	−	−	<1
−	+	−	−	<1
−	+	+	−	<1
+	+	−	−	18
+	+	+	−	40
+	+	+	+	43

[a] Enzyme polypeptide following gel filtration in the presence of $NaDodSO_4$ and subsequent removal of $NaDodSO_4$ by AG1-X2 in the presence of urea was added to 1 mL of 0.05 M Tris/acetate, pH 7.8, containing the indicated additions at final concentrations shown. Final [urea] = 0.29 M. [b] Partially purified soybean phospholipid. See Experimental Procedures for details. [c] Enzyme activity measured in a 1-mL assay containing 0.05 M Tes, pH 7.5, 250 μM NADH, 50 μM ubiquinone 1, and 40 μM FAD. Protein measured by Lowry method. Original specific activity of enzyme = 530 units mg^{-1}.

Tris/acetate buffer, pH 7.8. In both cases the maximum activity observed was ~50% of that reported in Table I.

Stereospecificity of E. coli Respiratory NADH Dehydrogenase. Essentially all enzymes that utilize nicotinamide nucleotides as redox coenzymes or substrates exhibit absolute stereospecificity with respect to transfer of hydride to or from the 4-position of the (dihydro)nicotinamide ring (Ryerson & Walsh, 1979; You et al., 1978; Simon & Kraus, 1976; Popják, 1970). Thus, enzymes that oxidize NAD(P)H catalyze exclusive removal of either the *pro-R* ("A") or *pro-S* ("B") hydrogen (as hydride) from the pair of diastereotopic methylene hydrogens at carbon 4 of the dihydronicotinamide moiety. This stereospecificity is a strongly conserved feature of different types of dehydrogenases during evolution (Bentley, 1970), apparently on account of the major rearrangement of active site groups that would be required to change a given enzyme's stereospecificity (Garavito et al., 1977).

Thus, the determination of the stereospecificity of a dehydrogenase with respect to the dihydronicotinamide ring may be expected to yield some information on the evolutionary relationships of the particular enzyme. In addition, it is a fundamental piece of information in building up a picture of the detailed mechanism of an enzyme.

For these reasons, we have examined the stereospecificity of the respiratory NADH dehydrogenase of *E. coli*, both in purified form, with ubiquinone 1 as acceptor, and in wild-type membranes. The nuclear magnetic resonance method (Arnold & You, 1978; Arnold et al., 1976) was used, and some results are shown in Table II. The stereospecificity of the purified enzyme was found to be 4-*pro-S*. A control experiment with yeast alcohol dehydrogenase, an enzyme of known 4-*pro-R* specificity, confirmed the specific 4*S* deuteration of the substrate (Table II).

The experiment with the purified NADH dehydrogenase in ^2H_2O as solvent (Table II) was performed to examine the possibility that the ^1H observed at the 4-position in ^1H_2O as solvent arose as a result of an exchange reaction between the ^2H in (4*S*)-[4-^{2}H]NADH and solvent. This possibility was eliminated by the results, which showed the complete retention of ^1H at the 4-position when the reaction was conducted in ^2H_2O (Table II).

The genetic evidence indicates that >98% of the NADH oxidase activity of crude, wild-type membranes proceeds via the respiratory NADH dehydrogenase flavoprotein which we have cloned, sequenced, and isolated (Young & Wallace, 1976; Young et al., 1978; Jaworowski et al., 1981a,b; Young et al., 1981). Thus, the stereospecificity of NADH oxidation by these membranes represents the stereospecificity of the respiratory NADH dehydrogenase flavoprotein in situ in native membranes. The stereospecificity of the wild-type membrane-bound NADH oxidase was demonstrated to be 4-*pro-S* (Table II), as for the purified enzyme.

In Table III, the stereospecificity of the *E. coli* respiratory NADH dehydrogenase is compared with the sterospecificities of a number of enzymes of interest. These are mainly reduced-pyridine-nucleotide-linked flavoprotein oxidoreductases, some of which will catalyze reduction of quinones.

Exchange Reaction with Solvent H_2O. The rate of exchange of the 4*S*-^{2}H of (4*S*)-[4-^{2}H]NADH with ^1H of solvent ^1H_2O catalyzed by the purified enzyme, in the absence of ubiquinone 1 as acceptor, was also examined. The results demonstrated that the rate of exchange was <0.6% of the reaction rate with ubiquinone 1 as acceptor [and (4*S*)-[4-^{2}H]NADH as substrate].

Discussion

In the present work, further amplification of the in vivo level of the respiratory NADH dehydrogenase of *E. coli* has resulted in membrane preparations which contain large amounts of the enzyme (10–15% of total protein). These membrane preparations are yellow on account of the highly elevated level of FAD which is also present. By quantification of the flavin and enzyme protein levels in these amplified membranes, we have been able to show that the enzyme in situ in crude membrane preparations contains 1 mol of FAD/mol of enzyme. We have also shown that the purified enzyme binds 1 mol of FAD/mol of enzyme (Jaworowski et al., 1981b).

Table II: Stereospecificity of *E. coli* Respiratory NADH Dehydrogenase for the Dihydronicotinamide Ring of NADH[a]

enzyme preparation	acceptor	solvent[b]	^1H at 4-position of nicotinamide ring in product NAD^+ (mol/mol)[c]	stereospecificity
purified NADH dehydrogenase	ubiquinone 1	^1H_2O	1.04	4*S*
purified NADH dehydrogenase	ubiquinone 1	^2H_2O	1.00	4*S*
wild-type membrane-bound NADH oxidase[d]	O_2	^1H_2O	1.00	4*S*
yeast alcohol dehydrogenase (4*R* specific)	acetaldehyde	^1H_2O	≤0.03	4*R*

[a] The substrate in all cases was (4*S*)-[4-^{2}H]NADH. [b] Buffered with 5 mM potassium phosphate, pH 7.5. [c] Determined by nuclear magnetic resonance spectrometry of product NAD^+ and corrected for isotopic purity of (4*S*)-[4-^{2}H]NADH (see text for details). [d] IY13 membrane vesicles.

Table III: Stereospecificity of Pyridine Nucleotide Oxidation by *E. coli* Respiratory NADH Dehydrogenase and Some Other Enzymes[a]

4-*pro*-*R* (A)	4-*pro*-*S* (B)
NADH:cytochrome b_5 reductase (microsomal)	respiratory NADH dehydrogenase[c] (*Escherichia coli*)
NADH dehydrogenase (mitochondrial, external)	respiratory NADH dehydrogenase (mitochondrial)
DT diaphorase [NAD(P)H dehydrogenase (quinone), rat liver]	glutathione reductase
NADPH oxidase[b] (polymorphonuclear leukocyte, superoxide-generating)	lipoamide dehydrogenase
NADH:FMN oxidoreductase (*Beneckea harveyi*)	thioredoxin reductase
Ferredoxin:$NADP^+$ oxidoreductase (spinach)	adrenodoxin reductase

[a] Unless otherwise specified, results are from Simon & Kraus (1976) or Arnold et al. (1978) and references therein. [b] Light et al. (1981). [c] This work.

Thomson & Shapiro (1981) found that their preparation of the respiratory NADH dehydrogenase had no visible absorption. However, they were dealing with a very low protein concentration (5 $\mu g\ mL^{-1}$), and the stimulation of the activity of their preparation of exogenous FAD suggests some loss of flavin during purification.

We have demonstrated that efficient ubiquinone-1 reduction catalyzed by the enzyme requires only the highly purified enzyme polypeptide, FAD, phospholipid, and the substrate NADH. Although Thomson & Shapiro (1981) have reported that the enzyme contains ~1 mol of Fe/mol of enzyme, we have definitively confirmed our previous finding that Fe is not involved (Jaworowski et al., 1981b). Indeed, the results of neutron activation analysis , together with the reconstitution of NADH:ubiquinone oxidoreductase activity from the enzyme polypeptide, strongly indicate that the respiratory NADH dehydrogenase of *E. coli* is not a metalloenzyme.

The stereospecificity of the respiratory NADH dehydrogenase of *E. coli* with respect to hydride abstraction from the 4-position of the dihydronicotinamide ring of NADH has been shown to be 4-*pro*-*S* (Table II). A number of enzymes under consideration in terms of possible evolutionary relationship with the *E. coli* NADH dehydrogenase are of opposite stereospecificity (Table III) and can hence be ruled out as having any close evolutionary relationship (Bentley, 1970; You et al., 1978; Garavito et al., 1977). These include, for example, NADH:cytochrome b_5 reductase, a membrane-bound flavoenzyme of similar subunit molecular weight and amino acid composition to the respiratory NADH dehydrogenase of *E. coli* (Young et al., 1981; Williams, 1976), as well as the NADPH oxidase of polymorphonuclear leukocytes (Light et al., 1981; Schirmer et al., 1983).

On the other hand, the stereospecificity of the *E. coli* respiratory NADH dehydrogenase is the same as that of the mammalian mitochondrial enzyme (Table III) which is consistent with (but does not establish) an evolutionary relationship between the two enzymes. It is known that the mammalian enzyme uses FMN (Hatefi & Stiggall, 1976; Galante & Hatefi, 1979), whereas the *E. coli* enzyme uses FAD, as do enzymes from the yeasts *Saccharomyces cervisiae* and *Candida utilus* (Duncan & Mackler, 1966; Mackler et al., 1981). Interestingly, while the *Candida utilus* enzyme utilizes FAD preferentially, it does also exhibit considerable activity with FMN (Mackler et al., 1981).

One further difference which has emerged from the present study is that the rates of exchange of the 4-*pro*-*S* dihydronicotinamide hydrogen with solvent in the absence of electron acceptors catalyzed by the *E. coli* and mammalian respiratory enzymes are different: the *E. coli* enzyme catalyzes exchange at a very low rate, whereas the mammalian enzyme catalyzes rapid exchange (Ernster et al., 1965; Lee et al., 1965). This could be related to the difference in the flavin cofactors (see below).

Recently it has been shown that glutathione reductase and lipoamide dehydrogenase exhibit extensive amino acid sequence homology with each other (Williams et al., 1982). They are similar in many other respects as well (Williams, 1976). Thioredoxin reductase also has some similarities, although there is no evidence of amino acid sequence homology in the vicinity of the active site disulfide group (Williams, 1976).

The complete amino acid sequence of human erythrocyte glutathione reductase has recently been reported (Krauth-Siegel et al., 1982). We have detected amino sequence homology between the *E. coli* respiratory NADH dehydrogenase and glutathione reductase (H. D. Campbell, I. G. Young, L.-S. Yeh, and M. O. Dayhoff, unpublished results). Consistent with this is the fact that the stereospecificities of glutathione reductase and the respiratory NADH dehydrogenase are the same (Table III). In addition, the rate of the exchange reaction with solvent catalyzed by *E. coli* glutathione reductase is low (Stern & Vennesland, 1960) as is the case with the *E. coli* respiratory NADH dehydrogenase. The low exchange rate could suggest that the isoalloxazine moiety is shielded from solvent (Popják, 1970) although other interpretations are possible. In the case of human glutathione reductase, where the three-dimensional structure has been determined at high resolution (Thieme et al., 1981), the FMN moiety of the FAD is completely buried within the enzyme (Schulz et al., 1982).

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Registry No. NADH, 58-68-4; (4*S*)-[4-^{2}H]NADH, 10021-11-1; NADH dehydrogenase, 9079-67-8.

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