Cheng, D. M., Danyluk, S. S., Dhingra, M. M., Ezra, F. S., MacCoss, M., Mitra, C. K., & Sarma, R. H. (1980) *Biochemistry 19*, 2591–2597.
Davies, D. B. (1976) *Stud. Biophys. 55*, 29–38.
Ezra, F. S., Lee, C. H., Kondo, N. S., Danyluk, S. S., & Sarma, R. H. (1977) *Biochemistry 17*, 1977–1987.
Giessner-Prettre, C., & Pullman, B. (1970a) *J. Theor. Biol. 27*, 87–93.
Giessner-Prettre, C., & Pullman, B. (1970b) *J. Theor. Biol. 27*, 341–342.
Giessner-Prettre, C., & Pullman, B. (1977) *J. Theor. Biol. 65*, 189–202.
Giessner-Prettre, C., & Pullman, B. (1978) in *Nuclear Magnetic Resonance Spectroscopy in Molecular Biology* (Pullman, B., Ed.) pp 147–161, Reidel, Dordrecht, The Netherlands.
Hogan, M. E., & Jardetzky, O. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6341–6344.
Lee, C. H., Ezra, F. S., Kondon, N. S., Danyluk, S. S., & Sarma, R. H. (1976) *Biochemistry 16*, 3627–3639.
Mitra, C. K., Dhingra, M. M., & Sarma, R. H. (1980a) *Biopolymers 19*, 1435–1450.
Mitra, C. K., Sarma, R. H., Giessner-Prettre, C., & Pullman, B. (1980b) *Int. J. Quantum Chem.* (in press).
Patel, D. J. (1979) in *Stereodynamics of Molecular Systems* (Sarma, R. H., Ed.) pp 397–435, Pergamon Press, Oxford and New York.
Patel, D. J., Canuel, L. L., & Pohl, F. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2508–2511.
Pohl, F. M. (1974) *Eur. J. Biochem. 42*, 495–504.
Pohl, F. M. (1976) *Nature (London) 260*, 365–366.
Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.
Pohl, F. M., Jovin, T. M., Baehr, W., & Holbrook, J. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3805–3809.
Prado, F. R., Giessner-Prettre, C., & Pullman, B. (1978) *J. Theor. Biol. 74*, 259–277.
Prado, F. R., Giessner-Prettre, C., & Pullman, B. (1980) *J. Mol. Struct.* (in press).
Sarma, R. H., Dingra, M. M., & Feldmann, R. J. (1979) in *Stereodynamics of Molecular Systems* (Sarma, R. H., Ed.) pp 251–264, Pergamon Press, New York.
Son, T-D., Guschlbauer, W., & Gueron, M. (1972) *J. Am. Chem. Soc. 94*, 7903–7911.
Wang, A. H-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.

Genetic Identification and Purification of the Respiratory NADH Dehydrogenase of *Escherichia coli*†

Anthony Jaworowski, Hugh D. Campbell, Maria I. Poulis, and Ian G. Young*

ABSTRACT: *Escherichia coli* membrane particles were solubilized with potassium cholate. An NADH:ubiquinone oxidoreductase was resolved by hydroxylapatite chromatography of the solubilized material. This enzyme has been identified as the respiratory NADH dehydrogenase since it is absent in chromatograms of solubilized material from an *ndh* mutant strain. Such mutants lack membrane-bound NADH oxidase activity and have previously been shown to have an inactive NADH dehydrogenase complex [Young, I. G., & Wallace, B. J. (1976) *Biochim. Biophys. Acta 449*, 376–385]. The respiratory NADH dehydrogenase was amplified 50- to 100-fold in vivo by using multicopy plasmid vectors carrying the *ndh* gene and then purified to homogeneity on hydroxylapatite. Hydroxylapatite chromatography of cholate-solubilized material from genetically amplified strains purified the enzyme ~800- to 1000-fold relative to the activity in wild-type membranes. By use of a large-scale purification procedure, 50–100 mg of protein with a specific activity of 500–600 μmol of reduced nicotinamide adenine dinucleotide oxidized min^{-1} mg^{-1} at pH 7.5, 30 °C, was obtained. Sodium dodecyl sulfate gel electrophoresis of the purified enzyme showed that the enzyme consists of a single polypeptide with an apparent M_r of 45000.

The respiratory NADH dehydrogenase catalyzes the transfer of electrons from NADH, generated mainly by glycolysis and the tricarboxylic acid cycle, to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. The electrons are passed ultimately to molecular oxygen, and ATP is synthesized via oxidative phosphorylation. The enzyme therefore links the major catabolic and energy-producing pathways in the cell. The mitochondrial enzyme has received considerable attention [e.g., see Ragan (1976) for a review], but, despite intensive investigation, very little is known about the mechanism of electron transfer, the energy conservation associated with it, or even the structure of the enzyme complex catalyzing these reactions.

Comparatively little work has been done with the corresponding enzyme from *E. coli* despite the advantages that genetic manipulation of this system can offer. In early attempts to solubilize and purify the enzyme from crude extracts (Wosilait & Nason, 1954; Bragg, 1965) or small particles (Kashket & Brodie, 1963; Bragg & Hou, 1967a,b; Gutman et al., 1968), no indications were given of the purity of preparations, nor were there adequate criteria to assign the activities purified to the respiratory dehydrogenase. Recently, Dancey et al. (1976) reported the purification of an NADH dehydrogenase from osmotically lysed membrane vesicles. The purified enzyme, which was composed of a single subunit with a molecular weight of 38000, could catalyze reduction of artificial electron acceptors such as potassium ferricyanide or dichlorophenolindophenol. Its activity toward ubiquinone was not reported.

We have previously isolated *E. coli* mutants (designated *ndh*) which have <2% of the wild-type level of membrane-bound NADH oxidase activity (Young & Wallace, 1976). The mutation was shown to affect the NADH dehydrogenase complex. By use of these mutants, the gene coding for the

†From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra City, A.C.T., 2601 Australia. *Received August 20, 1980.*

0006-2960/81/0420-2041$01.25/0

NADH dehydrogenase has been cloned onto amplifiable plasmids, and techniques were developed to increase the activity of this enzyme in the membrane over 50-fold (Young et al., 1978).

We now report the use of the mutant and plasmid strains in identifying the respiratory NADH dehydrogenase in cholate-solubilized preparations and the purification of NADH:ubiquinone oxidoreductase from "genetically amplified" strains. A technique for further amplifying the level of enzyme in the membrane is reported which allows convenient large-scale purification of the enzyme in a single step.

Experimental Procedures

Chemicals. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) was obtained from Vega Biochemicals (Tucson, AZ). Cholic acid was obtained from Fluka (Switzerland) and was twice recrystallized from 50% (v/v) aqueous ethanol by a procedure similar to that of Hatefi (1978). Stock 20% (nominal) solutions of potassium cholate, pH 7.5, were prepared by suspending 20 g of cholic acid in ~80 mL of water and titrating the mixture to pH 7.5 over several hours with concentrated KOH. The completely colorless solution was then made up to 100 mL, filtered, and stored at room temperature. In some early experiments, unrecrystallized cholate was used. Ubiquinone-1 was the generous gift of Drs. O. Isler (F. Hoffman-La Roche and Co., Basel, Switzerland) and D. Magrath. NADH and NADPH were from P-L Biochemicals (Milwaukee, WI), and FAD was from Sigma Chemical Co. (St. Louis, MO). *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, sodium salt (Tes), was obtained from Calbiochem (La Jolla, CA).

Growth Media. The mineral salts medium used has been described elsewhere (Stroobant et al., 1972) and was used at normal strength. The following supplements were sterilized separately and added at the indicated final concentrations to give the minimal medium: 30 mM mannitol, 0.1% (w/v) casamino acids (Difco), 0.15 mM L-histidine-HCl, 0.3 mM L-isoleucine, 0.3 mM L-valine, 0.2 mM L-tryptophan, and 1 μM thiamin (Young & Wallace, 1976). The complete medium used was brain–heart infusion (Oxoid). The selective plates for construction of IY85 contained the minimal medium (minus casamino acids), 2% (w/v) agar, 25 μg mL^{-1} ampicillin, and, where specified, 20 μg mL^{-1} X-gal (0.1 mL of a 4 mg mL^{-1} solution in dimethylformamide per plate.

Bacterial Strains and Plasmids. All strains used were derivatives of the *E. coli* K12 strains IY12 (*thi, his, ilv, trp, rpsL, ndh*) or its isogenic *ndh*$^+$ transductant IY13. Strains IY35 and IY85 carry the *ndh* plasmids pIY1 (Young et al., 1978) and pIY9, respectively, and were derived from strain IY12. The plasmid pIY9, derived from the vector pSF2124 (So et al., 1975), carries a double *lac* promoter inserted adjacent to the *ndh* fragment and was constructed from pIY1 as follows.

The plasmid pLJ3 (Johnsrud, 1978) was used as the source of an *Eco*RI fragment carrying a double *lac* promoter. This fragment was ligated into pIY1, which carries an *Eco*RI fragment containing the *ndh* gene. Plasmids pIY1 (3 μg) and pLJ3 (1.5 μg) were separately digested with *Eco*RI endonuclease and ligated in a total volume of 150 μL by using procedures previously described (Young et al., 1978). A 20-mL culture of IY12 (Klett 130) was transformed with the ligation mix by using the method of Lederberg & Cohen (1974), diluted 10-fold with complete medium, and grown overnight at 37 °C. Transformants were selected for resistance ampicillin and the ability to grow on mannitol as sole carbon source. These characteristics test for the presence of the vector pSF2124 and the *ndh* fragment, respectively. Viable colonies were then replated on selective plates containing X-gal. Colonies containing hybrid plasmids with the double *lac* promoter inserted are blue (Johnsrud, 1978). NADH-dependent ubiquinone-1 reductase and NADH oxidase activities were examined in membranes (see below) prepared from six such clones. The clone with the highest specific activity was designated IY85, and the plasmid it contains, pIY9.

Complementation of ndh Mutants by pIY1. Plasmid pIY1 was transferred from IY35 into strain C600 $r_k^-m_k^+$ by F-mediated conjugal transfer (Young & Poulis, 1978). The resulting derivative was then used as donor to transfer pIY1 into 11 independently isolated *ndh* mutants (Young & Wallace, 1976). Transconjugants were selected on complete medium and mannitol-minimal medium both containing ampicillin (25 μg mL^{-1}) and streptomycin (150 μg mL^{-1}). Equally high numbers of colonies on both plates indicated complementation of the *ndh* allele by pIY1.

Growth of Cells. All cultures were grown at 37 °C in minimal medium unless otherwise specified. Culture turbidity was measured by using a Klett–Summerson colorimeter fitted with a blue filter, and is expressed in Klett units.

Cells were harvested at Klett 200, using a Sharples continuous-flow centrifuge, unless otherwise specified. The cells were then washed (Wallace & Young, 1977), frozen in liquid N_2, and stored at −15 °C.

For screening membranes from different clones, 1-L cultures of cells were grown in 2-L baffled flasks and harvested by centrifugation for 10 min at 8000*g* in the GS-3 head of a Sorvall centrifuge.

For the preparation of membranes for small-scale column chromatography, 10-L batch cultures were grown in 14-L New Brunswick glass fermenters with aeration (12 L min^{-1}) and stirring (400 rpm). In experiments on this scale where the levels of enzyme were amplified by chloramphenicol treatment, growths were carried out as reported previously (Young et al., 1978).

For the large-scale enzyme purification, a 40-L batch culture of strain IY85 was grown in the stainless steel vessel of a New Brunswick Fermacell fermenter, Model CF-50, with aeration (30 L min^{-1}) and stirring (200 rpm). At Klett 200, 2 g of chloramphenicol was added and the culture incubated, typically for 8 h, under the same conditions as above. The cells were then harvested, resuspended in a small volume of sterile medium, and used to inoculate 9 × 10 L lots of fresh medium in the 14-L New Brunswick glass fermenters. The cultures were grown for one generation exactly as in the small-scale work and the cells harvested (yield ~150 g wet weight). The Fermacell was also used in the continuous culture mode to grow 240-L quantities of IY85 at Klett 200.

Preparation of Membranes. Membranes were prepared as described (Wallace & Young, 1977) except that the membrane pellet was collected by centrifugation for 2 h at 60 000 rpm in a Beckman 60 Ti rotor. The pellet in each 38-mL tube was mixed with 4.5 mL of STM buffer (0.25 M sucrose, 0.1 M Tes, and 0.02 M magnesium acetate, pH 7.5) and resuspended by gentle homogenization or, for large-scale work, subjected to three passes through a ground-glass syringe fitted with a 15-cm 18-gauge needle. Membranes were then washed in 2 volumes of STM buffer and the pellet, collected by centrifugation as before, was resuspended in the same buffer to 0.85 times the original volume of the membrane preparation. Membranes were frozen in liquid N_2 and stored at −15 °C.

Enzyme Assays. Procedures for the polarographic determination of succinate, D-lactate, and NADH oxidase activities

have been described previously (Young et al., 1978; Wallace & Young, 1977). One unit of oxidase activity in these assays is defined as the amount of enzyme catalyzing the uptake of 1 μmol of O min^{-1}.

NADH:ubiquinone oxidoreductase activity was measured at 30 °C by following ubiquinone-1-dependent NADH oxidation at 340 nm in a 1-mL reaction mixture containing 50 mM Tes buffer, pH 7.5, 250 μM NADH, 40 μM FAD, enzyme and 50 μM ubiquinone-1. The reaction was started by the addition of ubiquinone, and rates were calculated by using $\Delta\epsilon$ = 6810 M^{-1} cm^{-1} (Schatz & Racker, 1966). NADH oxidase activity was also measured spectrophotometrically by using the system described above, except that ubiquinone was not added and $\Delta\epsilon$ = 6220 M^{-1} cm^{-1}. Ubiquinone-1 was added as a 1 mM solution prepared by diluting ~20 mM ethanolic stock solutions in water. Stock ubiquinone-1 solutions were standardized spectrophotometrically as described previously (Crane & Barr, 1971). NADPH:ubiquinone oxidoreductase activity was measured as above with NADPH replacing NADH.

NADH:ferricyanide oxidoreductase activity was measured at 420 nm and 30 °C, using $\Delta\epsilon$ = 1000 M^{-1} cm^{-1}. The 1-mL reaction mixture contained 50 mM Tes buffer, pH 7.5, 250 μM NADH, 40 μM FAD, enzyme, and 1 mM $K_3Fe(CN)_6$. The reaction was started by addition of electron acceptor. When membranes were assayed for either NADH:ubiquinone or NADH:ferricyanide oxidoreductase activity, 3 mM KCN was included in the assay.

One unit of activity in the spectrophotometric assays is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NAD(P)H min^{-1}. Specific activity is defined as units per milligram of protein.

Purification of NADH:Ubiquinone Oxidoreductase. The following procedure was used to prepare highly purified enzyme from either chloramphenicol-amplified IY35 or IY85 membranes. Identical conditions were used to produce the column profiles for IY12 and IY13 membranes. All operations were performed at 0–4 °C. Membrane particles (12.5 mL) were washed and resuspended as described above, and then 1.9 mL of 20% cholate and 0.93 g of solid KCl were added to give final concentrations of 3% cholate and 1 M KCl. The material was mixed thoroughly by inversion, and insoluble material was pelleted by centrifugation for 3 h at 48 000 rpm in a Beckman SW56 rotor. The supernatant was loaded onto a column of hydroxylapatite (1.6 × 12 cm) equilibrated with 50 mL of 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% cholate and 20 μM FAD. A 400-mL linear gradient, 0.005–1 M potassium phosphate buffer, pH 7.5, containing 0.1% cholate and 20 μM FAD, was applied immediately at a flow rate of 30 mL h^{-1}. The NADH:ubiquinone oxidoreductase peak, which elutes toward the end of the gradient, was pooled, frozen in liquid N_2, and stored at −15 °C.

Large-Scale Enzyme Purification. Washed, resuspended membrane particles from one large scale growth of cells were placed in a beaker on ice, and 20% cholate and KCl were slowly added to the magnetically stirred solution to give final concentrations of 3% cholate and 1 M KCl. The mixture was centrifuged for 2 h at 60 000 rpm in a Beckman 60 Ti rotor. The supernatant above the brown layer was loaded onto a column of hydroxylapatite (5 × ~30 cm) equilibrated with 4 L of 20 mM potassium phosphate buffer, pH 7.5, containing 0.1% cholate and 20 μM FAD. A 4-L linear gradient, 0.02–1 M potassium phosphate buffer, pH 7.5, containing 0.1% cholate and 20 μM FAD, was applied immediately at a flow rate of 150–200 mL h^{-1}. Fractions of 20–25 mL were collected. The enzyme was frozen in liquid N_2 in 50-mL screwcap polypropylene centrifuge tubes and stored at −60 °C.

Concentration of Enzyme Solutions. Enzyme solutions were concentrated by ultrafiltration, using Amicon PM-10 membranes in an Amicon TCF-10 cell for large volumes, or appropriately sized stirred cells for smaller volumes.

Preparation of Hydroxylapatite. Hydroxylapatite was made from brushite essentially as described by Tiselius et al. (1956). The material showed large star-shaped crystals under the microscope with small platelike fines. Calcium and phosphorus analysis showed a Ca/P ratio of 1.44, similar to the ratio observed by Tiselius. The chromatographic properties of our preparations of hydroxylapatite were entirely reproducible in over three dozen enzyme purifications and were comparable to those of commerical preparations (Bio-Rad). Each column was used only once.

$NaDodSO_4$ Gel Electrophoresis. Electrophoresis on 10% and 15% $NaDodSO_4$ slab gels was performed by using the discontinuous buffer system of Laemmli (1970) as described previously (Young et al., 1978). Gels were also used in which the separation gel contained a linear 10–25% concentration gradient of acrylamide. For the determination of subunit molecular weight, the following proteins were used: γ-globulin (human), catalase (bovine liver), ovalbulin (chicken), L-lactate dehydrogenase (rabbit muscle), FDP-aldolase (rabbit muscle), hexokinase (yeast) pyruvate kinase (rabbit muscle), serum albumin (bovine), phosphoglyceromutase (rabbit muscle), myoglobin (horse muscle), lysozyme (chicken), ferritin (horse spleen), and cytochrome *c* (horse heart). All were of the highest grades commercially available. Molecular weight values for the standard proteins were obtained from Weber & Osborn (1969) and Darnall & Klotz (1975). Gels were scanned by using a Schoeffel Model SD 3000 spectrodensitometer at 540 nm with a slit width of 0.5 mm.

Inhibition Studies with Antibodies. Two rabbits were used for the preparation of antibodies to the purified NADH:ubiquinone oxidoreductase. For the first injection, pooled enzyme from hydroxylapatite chromatography (~1 mg in 0.4 mL of H_2O per rabbit) was emulsified with an equal volume of complete Freund's adjuvant and injected at multiple ventral sites. Subsequently, to ensure the maximum possible specificity of the resulting antibodies (Hartman & Udenfriend, 1969), purified enzyme was subjected to $NaDodSO_4$ gel electrophoresis as described above, except that thicker (0.3 cm) gels with wide sample wells were used. Gels were stained for 30 min at room temperature by using 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol and 10% (v/v) acetic acid and destained for 30 min in 5% (v/v) methanol and 10% (v/v) acetic acid. The protein band (~1 mL) was then excised, mixed with 0.5 mL of isotonic saline, homogenized with 1.5 mL of complete Freund's adjuvant, and injected as before. The first two such injections (~0.3 mg of protein per rabbit) were at 2-week intervals, and collection of blood from the ear vein was initiated after the second of these. Subsequent injections (~1 mg of protein per rabbit) were made monthly. A purified γ-globulin fraction was prepared from serum by ammonium sulfate precipitation as described by Chan & Schatz (1979). The final fraction was dialyzed against isotonic saline. Inhibition of enzyme activities was investigated as follows. Purified enzyme (2 μg of protein) was incubated with 0–300-μL purified γ-globulin in a total volume of 325 μL of isotonic saline for 10 min at room temperature prior to assay of aliquots for NADH:ubiquinone oxidoreductase activity. Wild-type (IY13) membranes (40 μg of protein) were incu-

Table I: Comparison of Purification of *E. coli* NADH:Ubiquinone Oxidoreductase from Wild-Type (IY13) and Genetically Amplified (IY35)[a] Membranes

step	volume (mL)		total activity (units)[b]		total protein (mg)		specific activity (units mg^{-1})	
	IY13	IY35	IY13	IY35	IY13	IY35	IY13	IY35
membranes	12.5	12.5	319	12 844	456	457	0.70	28.1
cholate soluble	10.2	10.5	72.0	12 093	181	127	0.40	95.2
hydroxylapatite	79.0	75.0	165	3 083	8.8	5.8	18.8	531

[a] Strain IY35 carries plasmid pIY1 bearing the *ndh* gene. For enzyme purification, the number of copoes of pIY1 was amplified by chloramphenicol treatment. See text for details. [b] NADH:ubiquinone oxidoreductase activity.

bated with 0–90 μL of purified γ-globulin in a total volume of 100 μL of isotonic saline for 15 min at room temperature prior to spectrophotometric assay of aliquots for NADH oxidase activity. Control γ-globulin was obtained from a rabbit injected with an unrelated antigen.

Protein Estimation. Protein was determined by the method of Lowry et al. (1951) using defatted bovine serum albumin (BSA) as standard. BSA stock solutions were standardized spectrophotometrically using $A^{1\%}_{1cm,280nm} = 6.7$. For column fractions, where the high phosphate concentration causes precipitation, the Lowry assay mix was centrifuged for 30 s at maximum speed in a bench centrifuge before reading. The A_{280} of column effluent was also monitored as a guide to protein concentration.

Results

Solubilization of NADH:Ubiquinone Oxidoreductase. Since the respiratory NADH dehydrogenase is believed to catalyze the transfer of electrons from NADH to ubiquinone-8 in vivo [see Wallace & Young (1977)], we wished to establish conditions of solubilization which did not destroy the ubiquinone reductase activity of the enzyme. A variety of solubilization procedures was therefore tested on membranes from wild-type strains to see whether a soluble preparation could be obtained which was active with ubiquinone-1 as electron acceptor. Ubiquinone-1 is considerably more water soluble than ubiquinone-8 and is therefore more suitable for spectrophotometric assays.

The best recovery of reasonably stable NADH:ubiquinone oxidoreductase activity was obtained by solubilizing membranes in 3% potassium cholate, pH 7.5, and 1 M KCl at 0–4 °C. Under these conditions, recoveries of activity were in the range 20–25%, with a slight decrease in specific activity (Table I). Solubilization of membranes in this manner followed by chromatography on hydroxylapatite (see below) revealed one major peak of ubiquinone reductase activity. The ubiquinone reductase peak (Figure 1a) was identified as the respiratory NADH dehydrogenase since it is absent from the profile of an analogous column on which solubilized membranes from the *ndh* mutant IY12 were chromatographed (Figure 1b). The respiratory NADH oxidase is lacking in *ndh* strains, and this is due to a mutation affecting the NADH dehydrogenase (Young & Wallace, 1976).

Amplification of NADH:Ubiquinone Oxidoreductase Levels. A hybrid plasmid (pIY1) containing the *ndh* gene has been isolated (Young et al., 1978). This plasmid was constructed from the multicopy cloning vector pSF2124 and *Eco*RI-digested *E. coli* chromosomal DNA. Plasmid pIY1 was obtained by selecting for complementation of an *ndh* mutant by a plasmid containing the *ndh* gene. It has been found that pIY1 complements all of the *ndh* mutants examined (see Experimental Procedures for details). The strain IY35, carrying pIY1, was found to overproduce the respiratory NADH dehydrogenase by 8- to 10-fold, an amount consistent with the gene copy number. Following the cloning of the *ndh*

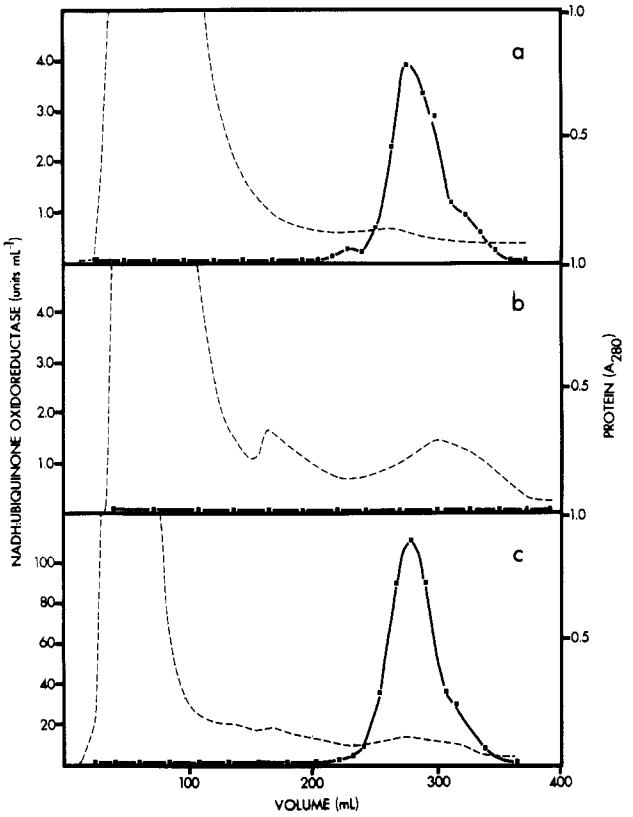

FIGURE 1: Hydroxylapatite chromatography of NADH:ubiquinone oxidoreductase from wild-type (a), *ndh* mutant (b), and plasmid-amplified (c) *E. coli* membranes. The strains used were (a) IY13, (b) IY12, and (c) chloramphenicol-amplified IY35. For each profile, 12.5 mL of membranes was washed, solubilized with cholate/KCl, and chromatographed on hydroxylapatite as described under Experimental Procedures. The A_{280} of the column effluent was continuously monitored (---), and the NADH:ubiquinone oxidoreductase activity of the fractions was measured (■).

gene, several approaches have been used to further amplify the level of the NADH dehydrogenase in the cell membrane.

In one approach (Young et al., 1978), the number of copies per cell of the hybrid plasmid carrying the *ndh* gene is increased by incubation of the cells with chloramphenicol, a protein synthesis inhibitor which stops cell division while allowing replication of the plasmid (Clewell, 1972). The chloramphenicol is subsequently removed, and the synthesis of NADH dehydrogenase occurs from the increased number of copies of the gene. By use of this approach, the level of the NADH:ubiquinone oxidoreductase can be amplified ~50- to 60-fold in the membrane without appreciable activity appearing in the cytoplasm (Table II). In IY35 membranes, the levels of the other respiratory activities D-lactate oxidase and succinate oxidase are relatively unaffected.

A second approach, reported in this paper, has involved the insertion of a fragment of DNA containing two copies of the strong *lac* promoter, presumably in appropriate orientation

Table II: Respiratory Activities[a] of Membrane and Cytoplasmic Fractions from *ndh*, Wild-Type and Genetically Amplified Strains

activity	IY12[b]	IY13[c]	IY35[d] (Cap. amp.)[f]	IY85[e]	IY85[e] (Cap. amp.)[f]
membrane fraction					
succinate oxidase	0.147	0.362	0.399	nd[g]	nd[g]
D-lactate oxidase	0.374	0.240	0.112	nd[g]	nd[g]
NADH oxidase[h]	<0.010	0.550	4.33	4.39	5.01
NADH:ubiquinone oxidoreductase	0.024	0.577	27.9	24.5	47.9
cytoplasmic fraction					
NADH oxidase[h]	0.042	0.041	0.077	0.097	0.276
NADH:ubiquinone oxidoreductase	0.679	0.718	0.635	1.96	2.47

[a] Values shown are specific activities (units mg^{-1}). See text for details. [b] *ndh* mutant. [c] Wild type. [d] Strain bearing hybrid plasmid pIY1. [e] Strain bearing hybrid plasmid pIY9. [f] Plasmid copy number amplified by chloramphenicol treatment. [g] Not determined. [h] Spectrophotometric assay.

with respect to the *ndh* gene on the hybrid plasmid, to form the plasmid pIY9. The strain IY85 carrying pIY9 overproduces the enzyme to a similar extent (~50-fold) to chloramphenicol-amplified IY35 (Table II). In this case, however, the higher yield of enzyme may be due not only to an increase in the gene copy number but also to increased efficiency of transcription. When strain IY85 was amplified with chloramphenicol, a further doubling of specific activity occurred, giving the maximum so far observed amplification of ~80- to 100-fold over the level in wild-type membranes, with a small but variable amount of activity appearing in the cytoplasm (Table II).

Purification of the NADH:Ubiquinone Oxidoreductase. Initial experiments on the purification of the respiratory NADH dehydrogenase were carried out on wild-type strains. As shown in Figure 1a, hydroxylapatite column chromatography separates the respiratory NADH dehydrogenase peak from the bulk of the protein. The separation is sensitive to the concentrations of cholate and FAD in the eluting buffer as well as the steepness of the gradient. Under optimum conditions a purification of 30- to 40-fold over the membrane-bound activity can be achieved in this single step, with an overall recovery of ~50% (Table I). The apparently low recovery on solubilization is probably the result of reversible inhibition of the enzyme activity by the high concentrations of cholate (A. Jaworowski, unpublished data). Such a drop in recovery is not found during purification of enzyme from genetically amplified strains, where, because of their very high activities, both membrane particles and solubilized material have to be diluted 100-fold prior to assay.

Attempts at further purification of the enzyme from hydroxylapatite chromatography resulted in large losses of activity. We felt that the excellent separation on hydroxylapatite could be coupled to the genetic amplification of the enzyme to give a high degree of purification. The amplified enzyme behaved identically on hydroxylapatite chromatography with the enzyme from wild-type cells (Figure 1c; cf. Figure 1a). The pooled enzyme after hydroxylapatite chromatography was purified 800- to 1000-fold relative to wild-type membranes (Table I). The enzyme migrates essentially as a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis (see below).

Large-Scale Purification of the NADH Dehydrogenase. Initially, large quantities of IY85 cells were grown on a 240-L scale in continuous culture. However, the specific activity of membranes obtained in this manner was only ~10 units mg^{-1}. Apparently this lowering of specific activity is a function of the continuous culture mode, since 40-L batch growth in the continuous culture vessel resulted in specific activities >20. Therefore, for large-scale growth of bacteria with high membrane specific activity, we resorted to chloramphenicol amplification of IY85, with the first stage of growth occurring in the 40-L stainless steel fermenter and the second stage in the 14-L glass fermenters. The preparation of membranes and hydroxylapatite chromatography are essentially scaled-up versions of these procedures.

Table III: Large-Scale Purification of *E. coli* NADH:Ubiquinone Oxidoreductase from Genetically Amplified (IY85)[a] Membranes

step	volume (mL)	total activity[b] (units)	total protein (mg)	specific activity	recovery (%)
membranes[c]	130	196 700	5 655	34.8	[100]
hydroxylapatite[d]	277	25 600	49.4	518	13.0

[a] Strain IY85 carries plasmid pIY9 bearing the *ndh* gene and a *lac* promoter fragment. For enzyme purification, the number of copies of pIY9 was amplified by chloramphenicol treatment. See text for details. [b] NADH:ubiquinone oxidoreductase activity. [c] Membranes were prepared from 204 g (wet weight) of chloramphenicol-amplified IY85 cells. [d] Washed membranes were solubilized with cholate/KCl. The supernatant (75 mL) was chromatographed on a 5 × 32 cm column of hydroxylapatite (Figure 3). Fractions 136–150 were pooled.

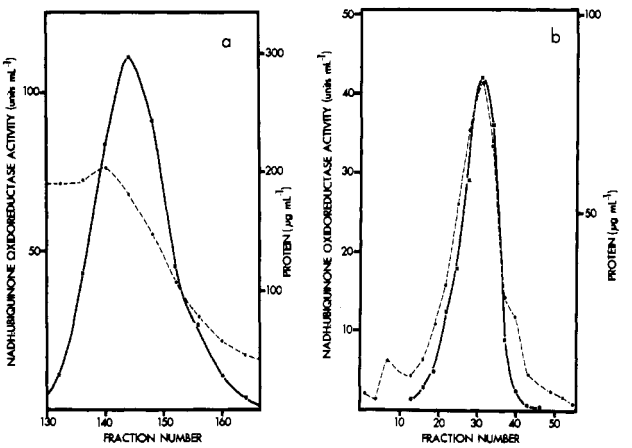

FIGURE 2: Large-scale hydroxylapatite chromatography of NADH:ubiquinone oxidoreductase from chloramphenicol-amplified IY85 membranes. (a) 130 mL of membranes was washed, solubilized with cholate/KCl, and chromatographed on a 4 × 32 cm column of hydroxylapatite as described in Table III and Experimental Procedures. (b) 110 mL of enzyme from (a) was concentrated to 12 mL by ultrafiltration, diluted 5-fold with 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% cholate and 20 μM FAD, and reconcentrated to 12 mL. This was repeated to achieve an overall dilution of 25-fold of the high phosphate concentration present from hydroxylapatite chromatography; 10.7 mL of this enzyme solution containing 14.0 mg of protein with an NADH:ubiquinone oxidoreductase specific activity of 540 units mg^{-1} was rechromatographed on a 1.6 × 12 cm column of hydroxylapatite exactly as in the small-scale purifications (Table I and Experimental Procedures). Fractions were assayed for protein by the Lowry method (●) and for NADH:ubiquinone oxidoreductase activity (■).

Results of a typical purification are shown in Table III. Figure 2a shows the hydroxylapatite chromatogram. The purification routinely yields 50–100 mg of enzyme protein with an NADH:ubiquinone oxidoreductase specific activity of 500–600 units mg^{-1} and an NADH:ferricyanide oxidoreductase specific activity of 130–150 units mg^{-1}. The ratio of activities toward ubiquinone-1 and ferricyanide as acceptors is similar (4:1) in both the wild-type (IY13) and plasmid-amplified enzymes after hydroxylapatite chromatography. Rechromatography of the purified enzyme on hydroxylapatite

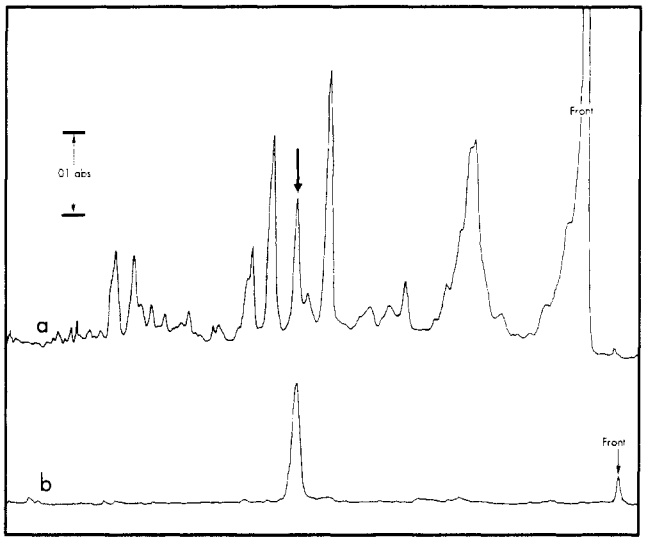

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of NADH:ubiquinone oxidoreductase from wild-type (a) and plasmid-amplified (b) *E. coli* membranes after hydroxylapatite chromatography. See Table I for the wild-type (IY13) preparation (a) and Table III for the large-scale purification of enzyme from chloramphenicol-amplified IY85 (b). Slab gels (10%) were run, stained, and scanned as described under Experimental Procedures. In scan a the arrow marks the peak corresponding in mobility to the purified enzyme (b). This was determined by experiments in which purified enzyme was added to the wild-type hydroxylapatite preparation prior to electrophoresis. The protein loads were (a) 60 μg and (b) 25 μg.

shows good coincidence between the protein and activity profiles, with no increase in specific activity (Figure 2b).

$NaDodSO_4$ gel electrophoresis reveals a single major band of protein in the purified enzyme (Figure 3b). An apparent M_r of 45 000 for this band was estimated by comparison with the mobilities of the protein standards on both 10 and 15% gels. This suggests that the protein is not anomalous in its $NaDodSO_4$-binding properties (Tanner, 1979) and that the apparent M_r of 45 000 is therefore close to the true M_r. There are traces of impurities of lower molecular weight present, as well as material migrating at the dye front on 10% gels (Figure 3b). The latter material was resolved into a diffuse group of very weakly staining bands of low molecular weight on a 10–25% gradient $NaDodSO_4$ gel when the gel was grossly overloaded (80 μg of protein/track), although these bands were not visible on a gradient gel at a normal load (8 μg of protein/track).

By using the purified enzyme as an internal standard, the corresponding band in the $NaDodSO_4$ gel of an hydroxylapatite preparation from wild-type membranes was identified (Figure 3). Assuming that the intensity of staining is proportional to the amount of protein, it was estimated densitometrically that this polypeptide represents ~3.4% of the protein on the gel. From this, and the specific activity of the preparation, it was calculated that the specific activity of the respiratory NADH dehydrogenase solubilized from wild-type membranes is ~560 units mg^{-1}, in good agreement with the value of 500–600 for the purified enzyme from genetically amplified strains.

Kinetic and Immunological Properties. The apparent K_m for NADH of the purified NADH:ubiquinone oxidoreductase under the assay conditions was estimated as ~57 μM from Lineweaver–Burk plots over the range 7–154 μM NADH. The apparent K_m of the purified enzyme for ubiquinone-1 was estimated as <5 μM. The NADH oxidase activity of wild-type (IY13) membrane particles exhibited an apparent K_m for NADH of ~55 μM, similar to the value obtained for the purified enzyme. The purified enzyme has no detectable activity with NADPH as substrate (specific activity <0.1 unit mg^{-1}).

Antibodies to the purified enzyme were prepared by using the M_r 45 000 species from $NaDodSO_4$–polyacrylamide gels to ensure specificity. These antibodies, while of low titre, inhibited both the NADH:ubiquinone oxidoreductase activity of the purified enzyme and the NADH oxidase activity of wild-type membranes a maximum of ~50% after taking the inhibition (maximum of 23%) caused by control antibodies into account.

Discussion

An NADH:ubiquinone oxidoreductase was solubilized from crude membrane particles with 3% potassium cholate and 1 M KCl at low temperature. Identification of this activity as the respiratory NADH dehydrogenase was achieved by genetic means. We have previously isolated mutant strains (designated *ndh*) which are totally deficient in the cyanide-sensitive, membrane-bound NADH oxidase. This has been shown to be due to a defective NADH dehydrogenase complex (Young & Wallace, 1976). The use of these mutants has allowed the cloning of the gene coding for this enzyme and the amplification of NADH:ubiquinone oxidoreductase levels in the membrane (Young et al., 1978, and this paper). By comparison of the hydroxylapatite column profiles of wild-type and *ndh*$^-$ strains, the respiratory NADH dehydrogenase can be definitely assigned as the major NADH-dependent ubiquinone-1 reductase peak (cf. Figures 1a,1b). This particular peak of activity is greatly amplified in the corresponding profiles of preparations from either chloramphenicol-amplified IY35 or IY85 cells, confirming that the gene coding for the respiratory enzyme has been cloned.

The respiratory NADH dehydrogenase is a relatively minor component of the cytoplasmic membrane of *E. coli*. The combination of genetic amplification and hydroxylapatite chromatography described above has given an 800-fold purification of the enzyme relative to wild-type membranes. These preparations of the enzyme consist of a single polypeptide of apparent M_r 45 000 and have a high specific activity. This is the first isolation of the *E. coli* respiratory NADH dehydrogenase in a pure form. Studies of the properties of the purified enzyme (Jaworowski et al., 1981) show that it contains one tightly-bound molecule of FAD per subunit and relatively large amounts of phosphatidylethanolamine. It can be used to reconstitute the NADH oxidase of membrane particles from an *ndh* mutant, suggesting that its ability to catalyze electron transport in vivo has not been affected by the purification employed.

Recently, several papers have been published describing other attempts made to isolate the respiratory NADH dehydrogenase of *E. coli* in a pure form. Dancey et al. (1976) reported the purification of an NADH dehydrogenase from *E. coli* which was active with a variety of electron acceptors including DCIP and ferricyanide, although its activity with ubiquinone was not reported. The most highly purified preparation contained ~75% of a single polypeptide of apparent M_r 38 000. The enzyme was purified 16-fold relative to the activity present after solubilization of spheroplasts with 5% (w/v) Triton X-100. The molecular weight of the major species is significantly lower than that of the enzyme described in the present work, and the activity of these preparations with ferricyanide as acceptor [Figure 2 of Dancey & Shapiro, (1976)] is some 300-fold lower. These two facts cast considerable doubt on the purity or the integrity of these preparations of the respiratory enzyme. In a more recent abstract,

Thomson & Shapiro (1979) have described a further preparation with somewhat different properties. The enzyme has two polypeptides of M_r 37 000 and 46 000 and has a specific activity with a short-chain ubiquinone analogue as acceptor of 75 μmol min^{-1} mg^{-1}. This is approximately 14% of the specific activity of the preparations described in this paper. It seems likely, therefore, that the component of M_r 46 000 corresponds to the respiratory enzyme which we have isolated in the present study.

The large-scale purification of the enzyme described here is simple and convenient and has been used in other studies to provide sufficient pure enzyme for detailed characterization. Protein chemical studies in conjunction with the determination of the DNA sequence of the cloned *ndh* gene have allowed the determination of the complete primary structure of the enzyme (Young et al., 1981). It consists of a single polypeptide of 433 amino acids with M_r 47 200. This value shows quite good agreement with the apparent molecular weight determined by $NaDodSO_4$ gel electrophoresis of the purified enzyme. Although the respiratory NADH dehydrogenase of *E. coli* performs a similar role to its counterpart in mitochondria (Ragan, 1976), it appears to be a considerably simpler enzyme. The isolation of the *E. coli* enzyme in a pure form should greatly facilitate a comparison with the components of complex I in mitochondria as they become better characterized.

Acknowledgments

We thank G. Mayo for excellent technical assistance and L. Johnsrud for a generous gift of plasmid pLJ3.

References

Bragg, P. D. (1965) *Biochim. Biophys. Acta 96*, 263–271.
Bragg, P. D., & Hou, C. (1967a) *Arch. Biochem. Biophys. 119*, 194–201.
Bragg, P. D., & Hou, C. (1967b) *Arch. Biochem. Biophys. 119*, 202–208.
Chan, S. H. P., & Schatz, G. (1979) *Methods Enzymol. 56*, 223–228.
Clewell, D. B. (1972) *J. Bacteriol. 110*, 667–676.
Crane, F. L., & Barr, R. (1971) *Methods Enzymol. 18*, 137–165.
Dancey, G. F., & Shapiro, B. M. (1976) *J. Biol. Chem. 251*, 5921–5928.
Dancey, G. F., Levine, A. E., & Shapiro, B. M. (1976) *J. Biol. Chem. 251*, 5911–5920.
Darnall, D. W., & Klotz, I. M. (1975) *Arch. Biochem. Biophys. 166*, 651–682.
Gutman, M., Schejter, A., & Avi-Dor, Y. (1968) *Biochem. Biophys. Acta 162*, 506–517.
Hartman, B. K., & Udenfriend, S. (1969) *Anal. Biochem. 30*, 391–394.
Hatefi, Y. (1978) *Methods Enzymol. 53*, 3–4.
Jaworowski, A., Mayo, G., Shaw, D. C., Campbell, H. D., & Young, I. G. (1981) *Biochemistry* (in press).
Johnsrud, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5314–5318.
Kashket, E. R., & Brodie, A. F. (1963) *J. Biol. Chem. 238*, 2564–2570.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lederberg, E. M., & Cohen, S. N. (1974) *J. Bacteriol. 119*, 1072–1074.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Ragan, C. I. (1976) *Biochim. Biophys. Acta 456*, 249–290.
Schatz, G., & Racker, E. (1966) *J. Biol. Chem. 241*, 1429–1438.
So, M., Gill, R., & Falkow, S. (1975) *Mol. Gen. Genet. 142*, 239–249.
Stroobant, P., Young, I. G., & Gibson, F. (1972) *J. Bacteriol. 109*, 134–139.
Tanner, M. J. A. (1979) in *Current Topics in Membranes and Transport* (Bronner, F., & Kleinzeller, A., Eds.) Vol. 12, pp 1–51, Academic Press, New York.
Thomson, J. W., & Shapiro, B. M. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2157 abstr.
Tiselius, A., Hjertén, S., & Levin, Ö. (1956) *Arch. Biochem. Biophys. 65*, 132–155.
Wallace, B. J., & Young, I. G. (1977) *Biochim. Biophys. Acta 461*, 84–100.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wosilait, W. D., & Nason, A. (1954) *J. Biol. Chem. 208*, 785–798.
Young, I. G., & Wallace, B. J. (1976) *Biochim. Biophys. Acta 449*, 376–385.
Young, I. G., & Poulis, M. I. (1978) *Gene 4*, 175–179.
Young, I. G., Jaworowski, A., & Poulis, M. I. (1978) *Gene 4*, 25–36.
Young, I. G., Rogers, B. L., Campbell, H. D., Jaworowski, A., & Shaw, D. C. (1981) *Eur. J. Biochem.* (in press).