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In Vitro Synthesis of the Respiratory NADH Dehydrogenase of *Escherichia coli*. Role of UUG as Initiation Codon†

Maria I. Poulis, Denis C. Shaw, Hugh D. Campbell, and Ian G. Young*

ABSTRACT: The respiratory NADH dehydrogenase of *Escherichia coli* has been synthesized in vitro in a coupled transcription–translation system with cloned deoxyribonucleic acid (DNA) as template. The identity of the protein produced was confirmed by paper chromatography and electrophoresis of tryptic peptides. [^{35}S]Methionine-labeled tryptic peptides from the in vitro product were shown to comigrate with authentic methionine-containing tryptic peptides from the purified enzyme. Using a transcription–translation system derived from an *ndh* mutant, it was shown that the enzyme produced in vitro was incorporated into membrane vesicles of the mutant to give functional, cyanide-sensitive NADH oxidase activity. Radiochemical N-terminal sequencing of the synthesized NADH dehydrogenase showed that the product was a mixture of three different species, with *N*-formylmethionine, methionine, or threonine at the N terminus. The results indicated that only partial N-terminal processing was occurring in vitro and that the first residue of the unprocessed NADH dehydrogenase is *N*-formylmethionine. Since DNA sequencing has shown that this residue is encoded by UUG [Young, I. G., Rogers, B. L., Campbell, H. D., Jaworowski, A., & Shaw, D. C. (1981) *Eur. J. Biochem.* (in press)], this work verifies the role of UUG as a normal initiation codon.

The respiratory NADH dehydrogenase of *Escherichia coli* is located together with the other components of the electron-transport chain in the inner or cytoplasmic membrane of this organism. It plays a central role in energy metabolism since it catalyzes the transfer of reducing equivalents generated by the major catabolic pathways to the membrane-bound energy-conserving system. The enzyme is a relatively minor, though highly active, component of the cytoplasmic membrane of *E. coli*, and this has made its purification difficult [see Jaworowski et al. (1981a)]. These difficulties have recently been overcome by the cloning of the *ndh*[1] structural gene and the amplification of the enzyme levels in vivo (Young et al., 1978). The enzyme has subsequently been purified to homogeneity and characterized (Jaworowski et al., 1981a,b).

We wished to produce the enzyme in vitro with the cloned DNA as template, in order to facilitate investigations into the regulation of synthesis of the NADH dehydrogenase, its mode of incorporation into the cytoplasmic membrane, and the structures of the unprocessed enzyme and any partially processed intermediates. The latter aspect has acquired particular significance because the determination of the sequence of the *ndh* gene and associated studies (Young et al., 1981) have suggested that a UUG codon, which normally specifies leucine, is acting as the translational initiation codon in this case. The initiating amino acid is efficiently removed in vivo by post-translational processing (Young et al., 1981).

In the present work, we describe the use of a coupled transcription–translation system to produce active, membrane-associated enzyme in vitro. The identity of the product

† From the Departments of Biochemistry (M.I.P., H.D.C., and I.G.Y.) and Physical Biochemistry (D.C.S.), John Curtin School of Medical Research, Australian National University, Canberra City, A.C.T. 2601, Australia. *Received October 23, 1980.*

[1] Abbreviations used: kbp, kilobase pairs; *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; TPCK, L-1-(*p*-toluenesulfonyl)amido-2-phenylethyl chloromethyl ketone.

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was demonstrated by protein chemical methods, and the putative role of UUG as a normal initiation codon specifying methionine is confirmed.

Experimental Procedures

Bacterial Strains and Plasmids. All strains used were derivatives of *E. coli* K12. Strain IY13 (*thi*, *his*, *ilv*, *trp*, and *rpsL*) is an isogenic *ndh*$^+$ transductant of the *ndh* mutant IY12 (Young et al., 1978; Young & Wallace, 1976). Strain IY59 carrying the hybrid plasmid pIY7 was constructed as follows. Plasmids pMB9 (Rodriguez et al., 1976) and pIY1, which possesses the 2.5-kbp *ndh* fragment cloned into the *Eco*RI site of pSF2124 (Young et al., 1978), were digested with *Eco*RI endonuclease and the products ligated as described previously (Young et al., 1978). Strain IY12 was transformed with the ligated DNA according to the method of Lederberg & Cohen (1974), and transformants were selected for resistance to tetracycline (10 μg mL^{-1}) and complementation of the *ndh* phenotype (Young & Wallace, 1976). One of these transformants (strain IY59) was shown to possess a hybrid plasmid (pIY7) carrying the 2.5-kbp *ndh* fragment cloned into the *Eco*RI site of pMB9.

Isolation of Plasmid DNA. Strain IY59 was grown in 2 × 1 L cultures on a glucose–mineral salts medium containing 0.5% casamino acids, and the plasmid DNA was amplified with chloramphenicol (Clewell, 1972). Plasmid DNA was extracted by the cleared lysate method of Clewell & Helinski (1969) except that 0.3% (w/v) Triton X-100 was the detergent used. The cleared lysate was extracted 3 times with chloroform–isopentyl alcohol (24:1 v/v), and the nucleic acids were precipitated with ethanol. The plasmid DNA was further purified by centrifugation through a CsCl–ethidium bromide density gradient (Clewell, 1972). Ethidium bromide was extracted with 2-propanol and the DNA dialyzed overnight at 4 °C against several changes of 10 mM Tris-HCl, 0.5 mM EDTA, and 10 mM NaCl, pH 7.4, before storage at −20 °C.

Purification of DNA Fragment Carrying the ndh Gene. The plasmid pIY7 was digested with *Eco*RI endonuclease to release the 2.5-kbp fragment carrying the *ndh* gene. This fragment was purified by agarose gel electrophoresis in the presence of ethidium bromide as described previously (Young et al., 1978), but using 1-cm-thick 0.8% agarose preparative gels. Agarose strips containing the 2.5-kbp fragment were embedded into a further 0.8% agarose gel, and the DNA was electrophoresed into a trough in the agarose gel filled with hydroxylapatite. The hydroxylapatite was removed and packed into a small plastic column, and the DNA was eluted with 0.4 M sodium phosphate buffer, pH 7.0. Ethidium bromide was removed by extraction with isopentyl alcohol, and the DNA solution was dialyzed overnight at 4 °C against 10 mM Tris-HCl, pH 7.4, containing 10 mM NaCl and 0.5 mM EDTA, and stored at −20 °C.

Coupled Transcription–Translation. The S-30 extract was prepared as follows: strain IY13 (or, where specified, IY12) was grown to mid-logarithmic phase in complete medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% glucose, 0.1% casamino acids, 0.2 mM L-histidine, 0.3 mM L-isoleucine, 0.3 mM L-valine, 0.1 mM L-tryptophan, and 3 μM thiamine). The cell pellet was washed with buffer A (10 mM Tris–acetate, 14 mM magnesium acetate, 60 mM potassium acetate, and 1 mM dithiothreitol, pH 7.8), then frozen in liquid N_2, and stored at −20 °C in 10-g lots. Cells were stored for up to 1 month prior to use.

Each lot of thawed cells was resuspended in 10 mL of buffer A and passed through a Ribi cell fractionator once at 20 000 psi or twice at 11 000 psi. Cell debris was removed by two consecutive 30-min centrifugations at 30000*g* in a Sorvall SE12 rotor at 4 °C. The top 75% of the second supernatant was taken and preincubated under the conditions described below, which are based on those used by Nirenberg (1963) and Whitfield & Bottomley (1980). The supernatant was mixed with a 1.66-mL solution (pH 7.8) containing 1 mmol of Tris–acetate, 25 μmol of magnesium acetate, 7 μmol of ATP, 80 μmol of phosphoenolpyruvate (potassium salt), 0.1 mg of pyruvate kinase (Boehringer), 1 μmol of GTP, 8 μmol of dithiothreitol, and 0.75 μmol of each of the 20 amino acids normally present in proteins, except methionine. The mixture was incubated for 2 h at 37 °C. The extract was then dialyzed for 4–6 h at 4 °C with three changes (each 3 L) of buffer A, centrifuged for 10 min at 30000*g* at 4 °C, and stored at −60 °C in small aliquots. The protein concentration of such preparations was 25–30 mg mL^{-1}.

The details of the coupled transcription–translation assay were given previously by Zubay et al. (1970). [^{35}S]Methionine (Amersham, 1000 Ci mmol^{-1}, 30 μCi/200 μL of reaction mixture) was used to label the polypeptide produced. The templates were used in the following quantities per 200 μL of reaction mixture: pIY7, 8 μg; isolated 2.5-kbp *ndh* fragment, 4 μg. Some changes to the composition of the reaction mixture described by Zubay et al. (1970) were made as follows. Poly(ethylene glycol) 6000 (1% w/v) was included in the reaction mixture, and the tRNA, pyridoxine hydrochloride, NADP$^+$, folinic acid, 4-aminobenzoic acid, and $CaCl_2$ were omitted. FAD was also omitted unless otherwise specified. For a 200-μL scale, the reaction was started by the addition of 40 μL of S-30 extract, and incubation was for 25 min at 37 °C. The reaction was generally terminated by the addition of cold acetone to a final concentration of 80% (v/v). The protein precipitate was collected by centrifugation and solubilized immediately as described below for $NaDodSO_4$–polyacrylamide gel electrophoresis. From 1 to 10% of the radioactivity was incorporated into trichloroacetic acid precipitable protein. If the membrane fraction was required, assay samples were diluted 40-fold in cold STM buffer (0.25 M sucrose, 0.1 M Tes, and 0.02 M magnesium acetate, pH 7.5) and centrifuged for 2.5 h at 50 000 rpm in a Beckman SW56 rotor at 4 °C. The pellet was solubilized for $NaDodSO_4$ gel electrophoresis as described below.

Detection of Activity of Enzyme Synthesized in Vitro. Coupled transcription–translation was carried out on a 25-mL scale with an S-30 extract derived from the *ndh* mutant IY12, in the presence and absence of pIY7 (25 μg mL^{-1}) as template. The reaction was as described above except that nonradioactive methionine was used at a final concentration of 0.1 mM, and pyridoxine hydrochloride, folinic acid (Ca salt), NADP$^+$, and FAD were included, all at 27 μg mL^{-1} final concentration. After the incubation, the reaction mixtures were each diluted to 70 mL with cold STM buffer and centrifuged at 60 000 rpm for 2 h in a Beckman 60 Ti rotor at 4 °C. The particulate fraction was resuspended in 3.5 mL of STM buffer in each case and assayed.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of $NaDodSO_4$ was performed on slab gels, using the discontinuous buffer system of Laemmli (1970) as described previously (Young et al., 1978), except that the separation gel usually consisted of a linear 10–25% concentration gradient of acrylamide. Samples were heated at 100 °C for 5 min in sample buffer (Laemmli, 1970) containing 5% (w/v) $NaDodSO_4$.

After being destained (Young et al., 1978), the gels were soaked in 30% (v/v) methanol for at least 30 min, dried under

heat and vacuum, and autoradiographed with Kodak X-Omat XS-5 X-ray film. Autoradiographs were scanned with a Schoeffel Model SD 3000 spectrodensitometer at 540 nm with a slit width of 0.5 mm.

Purification of Enzyme Polypeptide by Gel Filtration in $NaDodSO_4$. Purified enzyme from hydroxylapatite chromatography (Jaworowski et al., 1981a) was concentrated to 10 mg of protein mL^{-1} by ultrafiltration with an Amicon TCF10 ultrafiltration apparatus fitted with a PM10 membrane. The protein was then dialyzed at 4 °C against 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% cholate. To 3 mL of dialyzed enzyme at room temperature were added 225 μL of 2-mercaptoethanol and 1.5 mL of 20% (w/v) $NaDodSO_4$ with rapid mixing. The sample was incubated for 30 min at 60 °C, cooled to room temperature, and filtered through a 0.4-μm Millipore filter. The protein was immediately chromatographed at room temperature at a flow rate of 30 mL h^{-1} on a 2.6 × 65 cm column of Sephacryl S-300 equilibrated with 0.05 M NH_4HCO_3 containing 1% (w/v) $NaDodSO_4$.

Isolation of [^{35}S]Methionine-Labeled Tryptic Peptides. ^{35}S-Labeled NADH:ubiquinone oxidoreductase synthesized in vitro was purified by $NaDodSO_4$–polyacrylamide gel electrophoresis by using gradient gels similar to those described above. In the present case, no stacking gel was used, and the gels were prerun overnight in 0.375 M Tris-HCl, pH 8.8, containing 0.1% (w/v) $NaDodSO_4$ and 0.33% (w/v) 2-mercaptoethanol. Typically, acetone-precipitated protein from 1.5 mL of transcription–translation reaction mixture was heated for 5 min at 100 °C in 1.5 mL of 0.375 M Tris-HCl buffer, pH 8.8, containing 5% (w/v) $NaDodSO_4$, 10% (v/v) glycerol, and 0.33% (v/v) 2-mercaptoethanol, loaded onto two gels, and electrophoresed as described above with the normal electrode buffer (Laemmli, 1970). The gels were briefly rinsed in electrode buffer and dried immediately under heat and vacuum for autoradiography. The gels and autoradiograms were aligned and the radioactive protein bands excised. The dried gel slices were reswollen by addition of 8 mL of 0.05 M NH_4HCO_3, 1.0% (w/v) $NaDodSO_4$, and 1.0% (w/v) 2-mercaptoethanol containing 0.25 mg mL^{-1} pure, unlabeled NADH:ubiquinone oxidoreductase polypeptide, prepared as described above, as carrier. After 24-h incubation at room temperature, the supernatant was removed from the gel slices, which were then extracted with two further 8-mL lots of enzyme polypeptide (total, 6 mg of protein) as described above, except that no 2-mercaptoethanol was used. The extracts, stored at 4 °C, were combined, and the radioactive protein was concentrated to 2 mL by ultrafiltration through an Amicon PM10 membrane. Aliquots of the concentrated sample were checked for purity by electrophoresis on a gradient $NaDodSO_4$ gel, followed by protein staining and autoradiography as described above. The protein was freed of $NaDodSO_4$ by precipitation with 9 volumes of ice-cold acetone, collected by centrifugation in a bench centrifuge, washed twice with 90% (v/v) ice-cold acetone, and dried under vacuum at room temperature.

Labeled protein plus carrier, prepared as described above, was taken up in 2 mL of 0.063 M NH_4HCO_3. Trypsin (TPCK treated, 2% of weight of protein) was added and the solution incubated 4 h at 37 °C. Then 2-mercaptoethanol (1% v/v final concentration) was added and incubation continued for 1 h. Insoluble core material was pelleted by centrifugation, and the soluble tryptic peptide fraction was lyophilized prior to peptide mapping on Whatman 3MM paper as described previously (Bell et al., 1968). The first dimension was electrophoresis at pH 4.7 and the second dimension, ascending chromatography in butanol–acetic acid–pyridine–water, 15:3:10:12 by volume (plus 0.1% v/v 2-mercaptoethanol). Radioactive peptides were located by autoradiography at room temperature for two days as described above and were then subjected to further electrophoresis on paper at pH 1.9 (Bell et al., 1968). Autoradiography was used to locate the radioactive peptides and fluorescamine (Lai, 1977) to locate all peptides present in sufficient amounts. The radioactive peptides were excised, taking care, when necessary, to avoid nonradioactive contaminants, eluted, hydrolyzed for 24 h in 6 N HCl under vacuum, and analyzed on a Beckman 120C amino acid analyzer modified for single (6-mm) column operation.

N-Terminal Sequencing of Enzyme Synthesized in Vitro. [^{35}S]Methionine-labeled NADH:ubiquinone oxidoreductase was prepared as described above, by using a 2.5-mL transcription–translation mix with 50 μg of pIY7 as template. Unlabeled methionine (0.1 mM) was included in the assay to prevent any premature translational termination. The reaction was terminated by the addition of 1.2 mL of 20% (w/v) $NaDodSO_4$ and 188 μL of 2-mercaptoethanol and the mixture heated at 60 °C for 30 min. Unlabeled, purified NADH:ubiquinone oxidoreductase (3 mg) was treated with $NaDodSO_4$ in a similar fashion. The two samples were pooled, filtered through a 0.4-μm Millipore filter, and chromatographed on Sephacryl S-300 in the presence of $NaDodSO_4$ as described above. The unlabeled, authentic enzyme polypeptide of M_r 47 200 (Young et al., 1981) cochromatographed in this system with the radioactive polypeptide of apparent M_r 45 000, and these fractions were pooled. The column chromatography step gave essentially complete separation of the M_r 45 000 band from the lower molecular weight bands derived from pMB9 (see Figure 1). A 10-mg sample of bovine β-lactoglobulin A (Bell et al., 1968) was added to the pooled M_r 45 000 material to enable determination of repetitive yield on automated Edman degradation. The protein sample was then concentrated to 2 mL by ultrafiltration through an Amicon PM10 membrane and divided into two equal aliquots. The protein in each sample was precipitated with 90% acetone as described above. One sample was deformylated by suspending the dried protein in 2 mL of 0.1 M HCl in 50% (v/v) dioxane for 24 h at room temperature (Clark & Marcker, 1966a). This sample was then lyophilized.

The dried samples were redissolved in 8 M urea and subjected to reduction and alkylation as described by Crestfield et al. (1963), except that iodoacetamide was used. The samples were then dialyzed against 10% formic acid and then water, lyophilized, and redissolved in formic acid for application to the cup of a Beckman 890C sequencer. Samples were removed before loading for verification of the purity of the labeled protein by $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography as described above, and also for determination of total radioactivity. Automated Edman degradation (Edman & Begg, 1967) was performed by using a Beckman Quadrol protein program, No. 122974. The ^{35}S content of the anilinothiazolinones released at each step was determined directly by liquid scintillation counting. To check incorporation as methionine, and to determine the repetitive yield with the carrier β-lactoglobulin, we hydrolyzed the anilinothiazolinones to the free amino acids (Mendez & Lai, 1975; Walker et al., 1977) which were subjected to amino acid analysis as described above. The methionine peak was collected by diverting a fixed proportion (~90%) of the analyzer column effluent prior to ninhydrin addition into a fraction collector activated by an automatic integrator (Beckman Model 126 data system), essentially as described previously

(Walker et al., 1977; McKean et al., 1974). The samples were desalted as follows to improve counting efficiency. Each methionine-containing fraction of ~1 mL was acidified with 4 drops of 6 N HCl, diluted to 3 mL with H_2O, and applied to a small column of Dowex 50 (H^+ form) equilibrated with H_2O. The column was washed with H_2O, and the methionine was eluted with 1 M NH_3 and taken to dryness by rotary evaporation. The sample was then taken up in 2 mL of H_2O for liquid scintillation counting.

Enzyme Assays. NADH oxidase and NADH:ubiquinone oxidoreductase activities were assayed spectrophotometrically as described elsewhere (Jaworowski et al., 1981a). One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH min^{-1}. Specific activity is defined as units per milligram of protein.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) with defatted bovine serum albumin as standard.

Scintillation Counting. ^{35}S was measured by liquid scintillation counting in a Packard Tri-Carb 460 CD liquid scintillation spectrometer, using a xylene–Triton X-100 based scintillant.

Results

Synthesis of NADH Dehydrogenase Polypeptide in Vitro. The template DNA used routinely for these experiments was the plasmid pIY7. This plasmid consists of the 2.5-kbp DNA fragment carrying the *ndh* gene (Young et al., 1978) cloned into the *Eco*RI site of plasmid pMB9 and was constructed as described under Experimental Procedures. The coupled transcription–translation system used a 30000*g* supernatant (S-30) from *E. coli* strain IY13 and is described in detail under Experimental Procedures. [^{35}S]Methionine was used to label the translation products, which were then separated by NaDodSO$_4$–polyacrylamide gel electrophoresis and detected by autoradiography.

With pIY7 as template, analysis of the products showed that in addition to the low molecular weight bands attributable to pMB9 (Meagher et al., 1977) there was a strong band of apparent M_r 45 000 (Figure 1) which corresponded exactly in mobility to the purified enzyme (Jaworowski et al., 1981a) in 10%, 15%, and gradient NaDodSO$_4$–polyacrylamide gels. There was no indication of a form of the enzyme carrying a "signal" peptide. The difference in molecular weight caused by the presence of a transient hydrophobic leader sequence of 16–20 amino acids (Davis & Tai, 1980) would be expected to be detectable with the high-resolution discontinuous gel system used.

The S-30 used for the transcription–translation experiments still contains appreciable quantities of membrane fragments. Since the respiratory NADH dehydrogenase is exclusively membrane bound in vivo, it was of interest to determine whether the enzyme produced in vitro had become membrane associated. Accordingly, after incubation of a reaction mixture with pIY7 as template, the particulate fraction was separated by high-speed centrifugation. This fraction was shown to contain most of the enzyme band of M_r 45 000 (Figure 1), suggesting that the NADH dehydrogenase had become membrane associated in the in vitro system. Most of the pMB9-coded proteins synthesized in vitro were also membrane associated (Figure 1; Tait & Boyer, 1978).

Experiments were also carried out with the purified *ndh* fragment alone as template. Good synthesis of the NADH dehydrogenase was achieved by using the purified DNA fragment (Figure 1), indicating that the natural *ndh* promoter is present on the cloned fragment. This result is in agreement

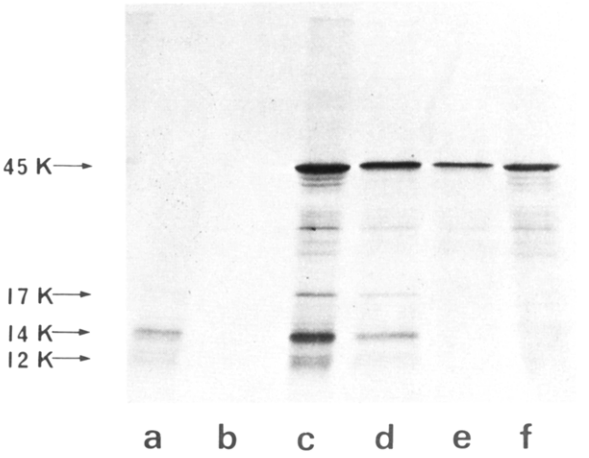

FIGURE 1: Autoradiograph of proteins synthesized in a coupled *E. coli* transcription–translation system in the presence of [^{35}S]methionine. Samples of the total assay mix and membrane fraction were electrophoresed on a 10–25% polyacrylamide gradient NaDodSO$_4$ gel as described under Experimental Procedures. Total proteins synthesized in the presence of various DNA templates are shown as follows: (a) the vector, plasmid pMB9 (10 μg/200 μL); (b) no DNA template added; (c) plasmid pIY7 (8 μg/200 μL); (f) cloned *ndh* fragment (4 μg/200 μL). Membrane-associated proteins synthesized from the templates are shown as follows: (d) plasmid pIY7 (8 μg/200 μL); (e) cloned *ndh* fragment (4 μg/200 μL). Arrows indicate the positions of purified, unlabeled NADH:ubiquinone oxidoreductase standard (apparent M_r 45 000) and pMB9 proteins (apparent M_r 17 000, 14 000, and 12 000).

with the results of DNA sequencing (Young et al., 1981), which show that the reading frame of the *ndh* gene commences 354 nucleotides in from the 5′ end of this fragment. Thus, the fragment would be expected to carry the *ndh* promoter (Rosenberg & Court, 1979).

Characterization of Enzyme Synthesized in Vitro by Peptide Mapping. In these experiments, the identity of the band of apparent M_r 45 000 was confirmed by peptide mapping. The labeled in vitro product was eluted from NaDodSO$_4$ gels and mixed with unlabeled, purified enzyme polypeptide. The mixture was then digested with trypsin, and the more soluble peptides were separated by two-dimensional peptide mapping on paper. The unlabeled peptides from the carrier NADH:ubiquinone oxidoreductase polypeptide were located by staining with fluorescamine and the [^{35}S]methionine-labeled peptides from the in vitro product by autoradiography. The essential identity (apart from possible terminal processing) of the two proteins was suggested by the fact that all but one of the major radioactive spots were exactly coincident with fluorescamine-positive spots (data not shown).

The major radioactive spots in a separate experiment were further purified by electrophoresis on paper at pH 1.9 to provide more conclusive evidence. The radioactive spots were eluted and their amino acid compositions determined. Seven spots were examined whose compositions (Table I) gave good agreement with those of methionine-containing tryptic peptides predicted from the DNA sequence of the *ndh* gene (Figure 2; Young et al., 1981). These peptides covered 11 out of the 14 methionine residues predicted to be present in the purified enzyme. These results provide good evidence that the methionine-containing peptides derived from the in vitro product are comigrating with those from the purified enzyme and that the proteins are therefore the same.

There was, however, one radioactive spot which varied in intensity in different experiments and which, as noted above, did not correspond to a fluorescamine-positive spot. Additionally, it did not analyze for Met (<1 nmol). This peptide

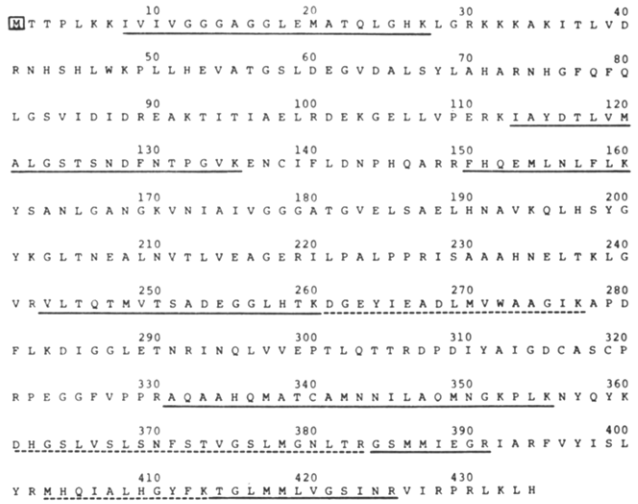

FIGURE 2: Primary structure of *E. coli* respiratory NADH dehydrogenase showing predicted Met-containing tryptic peptides. [^{35}S]Met-containing tryptic peptides identified in Table I are underlined with an unbroken line, and peptides not identified are underlined with a broken line. The sequence of residues 2–434 was derived from the nucleotide sequence of the *ndh* gene and confirmed from residues 2–20 by sequencing of the purified in vivo product (Young et al., 1981). Residue 1 (boxed) was identified as Met in the present work and is encoded by TTG in the nucleotide sequence. This residue is absent in the in vivo product. The one-letter code for amino acids (IUPAC–IUB Commission on Biochemical Nomenclature, 1968) is used.

was strongly basic and ran near a fluorescamine-positive spot identified as Thr-Thr-Pro-Leu-Lys-Lys, the N-terminal tryptic peptide derived from the purified enzyme (A. Jaworowski and D. C. Shaw, unpublished experiments; Young et al., 1981). The variable yield of this radioactive peptide suggested that it might arise as a result of incomplete N-terminal processing. Complete processing in vivo would explain the absence of a corresponding peptide from the purified enzyme. The position of migration of the spot is consistent with the peptide being Met-Thr-Thr-Pro-Leu-Lys-Lys, and the existence of such a species is supported by the N-terminal sequencing of the in vitro product (see below).

Amino-Terminal Sequence of the in Vitro Product. The amino-terminal sequence of the purified enzyme, which commences with Thr, has been determined and shown to agree with the amino acid sequence (Figure 2) predicted from the DNA sequence (Young et al., 1981). In the nucleotide sequence, the codon for the N-terminal Thr residue is immediately preceded by the codon UUG. UUG normally specifies Leu but in this case was assigned as the initiation codon because it was suitably positioned with respect to a good ribosome binding site (Shine & Dalgarno, 1974), and no appropriate AUG or GUG codons were present in the vicinity (Young et al., 1981). Since UUG has not previously been shown to be a normal initiation codon, it was necessary to verify its proposed role by structural studies on the unprocessed NADH dehydrogenase. Transcription–translation systems have generally been found to give less processing than that observed in vivo (Adams, 1968), so we investigated the N-terminal sequence of the in vitro product.

The in vitro product was prepared on a larger scale and purified by gel filtration in the presence of $NaDodSO_4$. This separated the enzyme from pMB9 translation products, which are of lower molecular weight, and the radiochemical purity of the enzyme preparations was verified by gel electrophoresis (Figure 3). The protein was deformylated, carboxamidomethylated, and subjected to automated Edman degradation as described under Experimental Procedures.

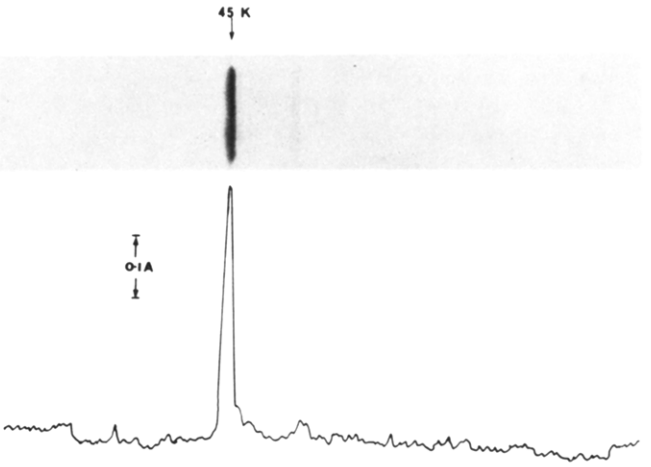

FIGURE 3: Autoradiograph of the in vitro radiolabeled polypeptide used for N-terminal sequence analysis. [^{35}S]Methionine-labeled NADH:ubiquinone oxidoreductase was prepared and treated as described under Experimental Procedures. A small aliquot of the undeformylated sample (2%, ~140 μg of protein including carrier) was electrophoresed on a 10–25% polyacrylamide gradient $NaDodSO_4$ gel, which was autoradiographed as described under Experimental Procedures. Also shown is a densitometric scan of the autoradiograph. Arrow indicates the position of purified, unlabeled NADH:ubiquinone oxidoreductase standard (apparent M_r 45 000).

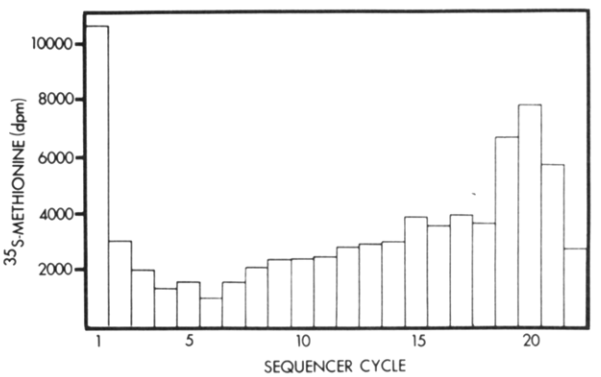

FIGURE 4: Automated Edman degradation of [^{35}S]methionine-labeled NADH:ubiquinone oxidoreductase synthesized in vitro. [^{35}S]-Methionine-labeled NADH:ubiquinone oxidoreductase produced in a large-scale coupled transcription–translation system with the *ndh* plasmid pIY7 as template was purified, mixed with β-lactoglobulin carrier, deformylated, and carboxamidomethylated. The protein was then subjected to automated Edman degradation with a Beckman 890C sequencer as described under Experimental Procedures. The counts shown are the total counts released at each sequencer cycle.

The ^{35}S counts liberated in the anilinothiazolinone fraction at each sequencer cycle are shown in Figure 4, and indicate Met at cycles 1, 19, and 20. The incorporation of ^{35}S as Met was confirmed by hydrolysis of the anilinothiazolinones to the free amino acids, followed by liquid scintillation counting of the Met peak from the amino acid analyzer. The same distribution of radioactivity was obtained as in Figure 4.

Direct N-terminal sequencing of the in vivo product (Young et al., 1981; H. D. Campbell and D. C. Shaw, unpublished experiments) has shown that the first 36 residues of the purified enzyme are as predicted from the DNA sequence. In particular, the only Met present in this region is at residue 19. The occurrence of Met at cycle 19 in the present work (Figure 4) is therefore attributed to the presence in the purified in vitro product of molecules with an N-terminal sequence identical with that of the purified enzyme. The additional presence of Met at cycles 1 and 20 in the case of the in vitro product indicates substantial quantities of an unprocessed product carrying one additional Met residue at the N terminus.

After correction of Met at cycle 20 for background (cycle

Table I: Amino Acid Composition of [^{35}S]Methionine-Labeled Tryptic Peptides from *E. coli* NADH Dehydrogenase[a]

	Tr1 (8–27)[b]	Tr2 (113–135)	Tr3 (150–160)	Tr4 (243–260)	Tr5 (331–355)	Tr6 (384–391)	Tr7 (414–425)
Asx		4.0 (4)	1.2 (1)	1.3 (1)	2.9 (3)		1.2 (1)
Thr	1.1 (1)[c]	4.1 (3)		3.8 (4)	1.4 (1)		0.6 (1)
Ser		2.1 (2)		0.9 (1)		1.1 (1)	1.3 (1)
Glx	2.2 (2)	1.1 (0)	2.2 (2)	2.1 (2)	3.4 (3)	1.3 (1)	
Pro		1.1 (1)			1.1 (1)		
Gly	5.5 (6)	2.5 (2)		2.1 (2)	1.3 (1)	1.9 (2)	1.9 (2)
Ala	2.0 (2)	2.4 (2)		1.2 (1)	5.8 (6)		
Val	1.4 (2)[e]	2.0 (2)		1.7 (2)			1.1 (1)
Met	0.7 (1)	0.8 (1)	0.8 (1)	0.8 (1)	2.3 (3)	1.7 (2)	1.0 (2)
Ile	1.1 (2)[e]	1.0 (1)			0.9 (1)	1.0 (1)	0.8 (1)
Leu	2.2 (2)	2.3 (2)	3.1 (3)	1.9 (2)	1.9 (2)		1.9 (2)
Tyr		0.9 (1)					
Phe		1.0 (1)	1.5 (2)				
Lys	1.1 (1)	0.9 (1)	1.3 (1)	1.1 (1)	1.9 (2)		
His	0.9 (1)		1.0 (1)	0.9 (1)	1.1 (1)		
Arg						1.0 (1)	0.7 (1)
Cys					nd[d] (1)		
amount recovered (nmol/residue)	6	9	4	35	5	23	5

[a] The NaDodSO$_4$ gel band of apparent M_r 45 000, labeled with [^{35}S]methionine in vitro, was mixed with purified, unlabeled NADH dehydrogenase polypeptide, and radioactive tryptic peptides were isolated and analyzed as described under Experimental Procedures. Values shown are normalized to give best fit to predicted compositions. Analytical values <2 nmol are not shown. [b] Residue numbering (inclusive) of predicted tryptic peptides in amino acid sequence (Young et al., 1981). [c] Values in parentheses are the integral number of each residue in the predicted tryptic peptides. [d] Not determined. [e] Low recovery attributed to incomplete hydrolysis of Ile-Val-Ile-Val sequence at residues 8–11.

18) and carryover from cycle 19, a repetitive yield of 93% was calculated, based on the recovery of Met at cycles 1 and 20. The same repetitive yield was obtained with the unlabeled carrier protein β-lactoglobulin, providing support for the occurrence of Met at residues 1 and 20 in a single sequence. The recovery of Met at cycle 1 indicated that ~50% of the in vitro product had N-terminal Met (or fMet), and the recovery of Met at cycle 19 was in good agreement with the remainder of the enzyme polypeptide starting with Thr.

The results of a sequencing experiment using the same material without the dioxane–HCl treatment gave a significantly lower recovery of Met at cycle 1 and indicated that ~40% of the N-terminal Met of the purified in vitro product was blocked by a group which is labile to mild acid hydrolysis, presumably an *N*-formyl group.

Enzymatic Activity of the in Vitro Product. We were interested in determining whether the enzyme made in vitro had normal enzymatic activity and could transfer electrons to the respiratory chain. Characterization of the in vivo product has shown the presence of one FAD per subunit. Apart from the noncovalently bound FAD, and the possibility that the enzyme may require phospholipid for activity, no other prosthetic groups have been identified (Jaworowski et al., 1981b). Since the flavin is not covalently bound, this requirement was easily satisfied by addition of FAD to the transcription–translation system.

For these experiments, it was essential to use an S-30 extract prepared from an *ndh* mutant so that the activity of the synthesized enzyme could be monitored by examination of the membrane fraction. Membrane vesicles prepared from the *ndh* mutant IY12 have <2% of the wild-type level of NADH oxidase activity, and this residual activity is cyanide insensitive. It was not possible to examine the soluble fraction for de novo synthesized NADH:ubiquinone-1 oxidoreductase activity since several soluble enzymes have appreciable activity in the assay with ubiquinone-1 as acceptor. In any case, previous experiments had indicated that the majority of the synthesized enzyme was in the membrane fraction (Figure 1).

The results in Table II show that there was a 20-fold increase in NADH:ubiquinone oxidoreductase activity in the membrane fraction of the S-30 from IY12 when pIY7 was used as template. The amount of enzyme produced restored the enzyme activity of the membranes to double the wild-type level (Young et al., 1978). There was also a corresponding large increase in the level of NADH oxidase activity, which, like the wild-type activity, was inhibited more than 90% by cyanide (Table II). This suggests that the de novo synthesized NADH dehydrogenase is transferring electrons to oxygen via the cyanide-sensitive cytochrome oxidase system. The only ubiquinone present in the oxidase assay is the endogenous ubiquinone-8. Since the ratio of NADH oxidase to NADH:ubiquinone-1 oxidoreductase activity (0.57, Table II) obtained with the de novo synthesized activity is within the range encountered with the wild-type activities, it is likely that all or most of the membrane-associated enzyme which is active in the reduction of ubiquinone-1 can transfer electrons to the respiratory chain.

Table II: Activity of NADH Dehydrogenase Synthesized in Vitro[a]

DNA template for in vitro synthesis	sp act. of membrane fraction[b] NADH:ubiquinone oxidoreductase[c]	NADH oxidase (–KCN)	NADH oxidase (+KCN)[c]
none	0.05	0.03	0.03
pIY7	1.18	0.67	0.06

[a] The S-30 used for coupled transcription–translation was derived from the *ndh* mutant IY12. Plasmid pIY7, which carries the *ndh* structural gene, was used as template. After in vitro synthesis, the membrane fraction was isolated by centrifugation and assayed for NADH:ubiquinone oxidoreductase and NADH oxidase activities. [b] Specific activities are given as micromoles of NADH oxidized per minute per milligram of protein at 30 °C. [c] Assayed in the presence of 3 mM KCN.

Discussion

The transcription–translation experiments show that the 2.5-kbp cloned fragment in plasmid pIY7 carries the structural gene and promoter region for the respiratory NADH de-

hydrogenase of *E. coli*. The enzyme can be efficiently synthesized in vitro, and most of the product becomes membrane associated. The use of an in vitro system derived from an *ndh* mutant strain has allowed the demonstration that the enzyme made in vitro is active and can efficiently reconstitute the NADH oxidase activity of the mutant. These observations suggest that following its synthesis the NADH dehydrogenase is correctly inserted into the membrane vesicles present in the transcription–translation system. This system should prove valuable for further work concerning the regulation of expression of the *ndh* gene.

The mechanism by which the NADH dehydrogenase becomes membrane associated is unknown. The DNA sequence of the structural gene and the N-terminal sequence of the in vivo product have been determined (Young et al., 1981), and, in agreement with the present work, there is no indication of the use of a signal peptide analogous to those found with various membrane proteins, secreted proteins, and proteins transported to the outer membrane or periplasmic space of *E. coli* (Davis & Tai, 1980). Not all membrane proteins have signal peptides, however, so such a structure is not necessarily expected in this case. The purified NADH dehydrogenase (Jaworowski et al., 1981a) has been shown to readily become membrane associated and to reconstitute cyanide-sensitive NADH oxidase activity when simply added to an aqueous suspension of *ndh* mutant membrane vesicles (Jaworowski et al., 1981b). This indicates that the mature enzyme can become membrane associated by a mechanism not dependent upon concomitant protein synthesis. It should be noted that the purified enzyme preparations used in the reconstitution contain ~70% phospholipid, which may facilitate incorporation of the protein into the membrane.

The amino-terminal sequence of the enzyme found in vivo starts with a threonine residue corresponding to an ACT codon at nucleotides 357–359 of the cloned *ndh* fragment (Young et al., 1981). This indicated that removal of the first residue or residues occurs by posttranslational processing. This has been confirmed in the present work by the amino-terminal sequencing of the in vivo product. The incomplete processing occurring in vitro has allowed the detection of precursor forms of the NADH dehydrogenase, beginning with either methionine or *N*-formylmethionine as the N-terminal residue. Since the gene sequence (Young et al., 1981) shows that this residue is encoded by UUG, the work presented here verifies that a UUG triplet is the translational initiation codon in the NADH dehydrogenase gene, where it specifies *N*-formylmethionine. Normally, UUG specifies internal leucine residues.

Together with AUG, GUG, and GUA, UUG was implicated as an initiation codon in early experiments which measured the efficacy of various trinucleotides in promoting the binding of $tRNA^{fMet}$ to ribosomes. However, of these four codons, UUG alone was discounted because it was inactive as an initiation codon in protein synthesis from synthetic polynucleotide templates in vitro (Ghosh et al., 1967; Clark & Marcker, 1966b).

Subsequent work has shown that AUG is the usual in vivo initiation codon (Grunberg-Manago & Gros, 1977). GUG has been shown to be the initiation codon for two proteins so far, the A protein of bacteriophage MS2 (Volckaert & Fiers, 1973; Fiers et al., 1975) and the *E. coli lac* repressor protein (Steege, 1977; Farabaugh, 1978). While the codon GUA was considered a likely initiation codon in the early in vitro studies (Ghosh et al., 1967), there is no documented instance of its use as an initiation codon in vivo. Previous studies on translational reinitiation after premature termination by amber mutations in the *lac* repressor gene have shown that UUG can act as a reinitiation codon in vivo, in which case it codes for methionine (Farabaugh, 1978; Files et al., 1975; Ganem et al., 1973). The *E. coli* NADH dehydrogenase system represents the first case in which UUG has been demonstrated to act as a normal initiation codon in vivo.

Acknowledgments

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Laser Photochemically Induced Dynamic Nuclear Polarization Proton Nuclear Magnetic Resonance Studies on Three Homologous Calcium Binding Proteins: Cardiac Troponin-C, Skeletal Troponin-C, and Calmodulin†

Maxwell T. Hincke,‡ Brian D. Sykes, and Cyril M. Kay*

ABSTRACT: Laser photo-CIDNP ^{1}H NMR experiments were performed with rabbit skeletal troponin-C (sTn-C), bovine cardiac troponin-C (cTn-C), and bovine brain calmodulin to study the exposure of histidine and tyrosine residues. In cTn-C, tyrosine residues, 5, 111, and 150 were exposed in the apoprotein, becoming buried as Ca^{2+} was bound. A similar phenomenon was observed for tyrosine residues 10 and 109 of sTn-C. In calmodulin, only tyrosine-99 was accessible in the apoprotein. The lack of exposure of tyrosine-138 observed with this technique correlates with the buried nature of this residue implied by other criteria. In 6 M urea each of the apoproteins was observed to be unfolded from the standpoint of the tyrosine environments. A large tyrosyl CIDNP effect was obtained for each protein which decreased as Ca^{2+} was bound, with a stoichiometry of one metal ion per protein. This was correlated for cTn-C with the appearance of "native" resonances representing tyrosine residues 111 and 150 in Ca^{2+}-saturated cTn-C, also with a stoichiometry of one. Analysis of our NMR findings, in the light of other spectroscopic and model building studies on these systems, suggests that the sole high-affinity Ca^{2+} binding site of cTn-C and sTn-C remaining in 6 M urea is site IV.

Troponin-C, from beef cardiac (cTn-C)[1] and rabbit skeletal muscle (sTn-C), and bovine brain calmodulin are calcium binding proteins which have been extensively characterized by ^{1}H NMR spectroscopy [Hincke et al. (1981), Seamon et al. (1977), and Seamon (1980), respectively]. These studies have assigned various features of the spectra, especially the tyrosine and histidine resonances, to specific residues within the primary sequence and have used the assigned resonances to monitor the large conformational changes which occur in these proteins upon Ca^{2+} binding.

These proteins are of particular interest because of their biological importance. Tn-C is the Ca^{2+} binding subunit of the troponin complex in vertebrate striated muscle. Ca^{2+} binding initiates a chain of molecular events which leads to activation of the Mg–ATPase of actomyosin and contraction (McCubbin & Kay, 1980; Perry, 1979). Calmodulin is a protein widely found in nonmuscle tissue [see Cheung (1980) for a review] which activates numerous enzymes by complexing with them only when Ca^{2+} is present. Some well-studied examples are 3′,5′-cyclic nucleotide phosphodiesterase (Techima & Kakiuchi, 1974) and adenylate cyclase (Lynch et al., 1976).

A technique has recently been introduced which allows NMR signals to be selectively detected from histidine, tyrosine, and tryptophan residues which are on the protein surface. This method is based on the laser photochemically induced dynamic nuclear polarization (photo-CIDNP) of the aromatic protons of these residues (Kaptein, 1978). A flavin dye, excited to its triplet state by brief laser irradiation, reacts reversibly with any accessible histidine, tyrosine, or tryptophan residues that

† From the Medical Research Council Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. *Received December 11, 1980.* M.T.H. was supported by a studentship from the Medical Research Council of Canada.

‡ Present address: CRBM du CNRS, B.P. 5051, Route de Mende, 34033 Montpellier, France.

[1] Abbreviations used: photo-CIDNP, photochemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; FMN, flavin mononucleotide; cTN-C, bovine cardiac troponin-C; sTN-C, rabbit skeletal troponin-C; MCBP, muscle calcium binding protein (parvalbumin); Mops, morpholinopropanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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