Biochem J. (1980) **191**, 457–465
Printed in Great Britain

Purification of an NADH–(dichlorophenol-indophenol) oxidoreductase from *Bacillus stearothermophilus*

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(*Received 24 March 1980/Accepted 26 June 1980*)

An NADH–(dichlorophenol-indophenol) oxidoreductase was purified 104-fold and in 25% overall yield from the thermophilic bacterium *Bacillus stearothermophilus*, strain PH24. After solubilization in 2M-NaCl at 70°C, the enzyme was purified by ion-exchange and hydroxyapatite chromatography, followed by affinity chromatography on immobilized Cibacron Blue 3GA. The purified enzyme had a mol.wt. of 43000 and had an absorption spectrum characteristic of flavoprotein. The enzyme activity was enhanced by FMN and by CN^-. The enzyme was inhibited by EDTA and by *p*-chloromercuribenzoic acid.

The NADH dehydrogenase [NADH–(2,6-dichlorophenol-indophenol) oxidoreductase, EC 1.6.99.3] from mammalian mitochondria has been the focus of intensive study during the past two decades, as a result of its function as the main electron entry point in the respiratory chain (Singer, 1968; Singer & Gutman, 1971). The respiratory-chain-linked NADH dehydrogenases of bacteria have not received as much attention as their mammalian counterparts, and relatively few have been purified (Dancey *et al.*, 1976; Hochstein & Dalton, 1973; Jinks & Matz, 1976).

However, the purification of two distinct NADH dehydrogenases from the thermophilic bacterium *Bacillus stearothermophilus* has been reported (Atkinson, 1973), although no data on the purities and properties of the two enzymes were given. Preliminary studies with an NADH dehydrogenase of *B. stearothermophilus* implicated this enzyme as the first enzyme in the NADH oxidase pathway of electron transport of the bacterial cell (Mains, 1978). Accordingly a more rigorous purification and characterization of the NADH dehydrogenase activity of *B. stearothermophilus* was attempted.

Materials and methods

NADH, NAD^+, FAD, FMN, riboflavin, bovine serum albumin, trypsin, pepsin, ox heart cytochrome *c*, AMP and EDTA were all purchased from Sigma Chemical Co. Dichlorophenol-indophenol, SDS, acrylamide, *NN'*-methylenebisacrylamide and *p*-chloromercuribenzoate were obtained from BDH Ltd., Poole, Dorset, U.K. Whatman DE-52 ion-exchange DEAE-cellulose was purchased from Whatman, Maidstone, Kent, U.K. Sephadex G-50 (medium grade), G-200 (superfine grade) and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Cibacron Blue 3GA was a gift from Ciba–Geigy Ltd., Dyestuffs Division, Manchester, U.K.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Growth of bacteria and preparation of cell-free extracts

B. stearothermophilus strain PH24 was grown at 55°C on a mineral-salts medium (Buswell, 1975), with phenol as the major carbon source. Cells were harvested towards the end of the experimental phase (about 14h). After being washed once with 0.1M-potassium phosphate buffer, pH 7.5, the cells were suspended in 2vol. of the same buffer and broken by four passages through an X-press (AB Biox, Nacka, Sweden) previously cooled to −20°C. After treatment with bovine pancreatic deoxyribonuclease (10μg/ml) for 15min at 23°C, unbroken cells and cell debris were removed by centrifugation at 30000*g* for 20min (Buswell, 1975). The supernatant material, termed the crude extract, was used for the purification of the NADH dehydrogenase.

Enzyme assay

NADH dehydrogenase activity was assayed in a

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Pye–Unicam SP. 1800 recording spectrophotometer with the cell compartment thermostatically maintained at 55°C. Cuvettes contained: 0.5M-potassium phosphate buffer, pH 7.5 (2.0ml); 0.01% (w/v) dichlorophenol-indophenol (0.3ml); 1% (w/v) NADH (0.15ml); sufficient test sample to give ΔA_{600} of 0.1–0.2/min, and distilled water to give a total volume of 3.0ml. Observed rates were corrected for non-enzymic reduction of dichlorophenol-indophenol by NADH. One unit of NADH dehydrogenase activity is defined as the amount causing reduction of 1μmol of dichlorophenol-indophenol/min at 55°C and pH 7.5. ε_{600} of 21.0×10^3 litre·mol^{-1}·cm^{-1} was assumed for dichlorophenol-indophenol (Dancey *et al.*, 1976). Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Specific activity is expressed as units of enzyme activity per mg of protein.

Media for adsorption and chromatography

Hydroxyapatite was prepared by the method of Atkinson *et al.* (1973), the final washed precipitate being stored in 5mM-potassium phosphate buffer, pH 6.8.

Whatman DE-52 anion-exchange DEAE-cellulose was purchased pre-swollen, and was washed extensively with 50mM-Tris/HCl buffer, pH 7.5, before use. Gradient elutions were performed with a commercially available (Pharmacia) gradient mixer.

Affinity-chromatography medium

Immobilized Cibacron Blue 3GA was prepared by a modification of the method of Heyns & DeMoor (1974). To Sepharose 4B (50ml packed volume) was added 500mM-Na_2CO_3 solution (17ml), followed by Cibacron Blue 3GA (0.33g in 17ml of distilled water). The mixture was incubated at 45°C for 50h with occasional stirring. Excess dye was removed by extensive washing with distilled water on a sintered-glass funnel, and the resulting gel stored at 4°C under 5mM-potassium phosphate buffer, pH 7.5, containing 0.1% (w/v) NaN_3.

Polyacrylamide-gel electrophoresis

Purity of the NADH dehydrogenase preparation was determined by polyacrylamide-gel electrophoresis by the method of Davis (1964), but no stacking gel was used. The separating gels were composed of 7.5% acrylamide and 0.2% *NN'*-methylenebisacrylamide in 7.5cm gel tubes. The running buffer was 0.025M-Tris/0.19M-glycine, pH 8.3. The purified enzyme preparation (20μl; 35μg of protein) was electrophoresed at a constant current of 4mA/gel tube and temperature of 10°C. After electrophoresis, the gels were stained for protein with Amido Black. Gels selected for NADH dehydrogenase assay were not stained, but cut into small sections (5mm), each section being homogenized in distilled water and assayed for enzyme activity.

Molecular-weight determinations

(*i*) *SDS/polyacrylamide-gel electrophoresis.* This was performed by the method of Weber & Osborn (1969) in a gel of the same composition as described above but containing 0.1% (w/v) SDS. Electrophoresis was performed at a constant current of 10mA/tube with bovine serum albumin, pepsin, trypsin and ox heart cytochrome *c* as marker proteins. Approx. 80μg of purified NADH dehydrogenase was used. Gels were stained with Amido Black.

(*ii*) *Gel filtration.* Gel filtration was performed as described by Andrews (1965). A 0.9cm × 60cm column of Sephadex G-200 (superfine grade) (the void volume was determined by using Blue Dextran) pre-equilibrated with 50mM-potassium phosphate buffer (pH 7.5) was eluted at a flow rate of 1ml/h. Bovine serum albumin, trypsin and pepsin were used as marker proteins.

Ultrafiltration

Protein samples were concentrated by ultrafiltration with an Amicon model 52 ultrafiltration system with UM10 membranes (Amicon, High Wycombe, Bucks., U.K.).

Measurement of absorption spectrum

An absorption spectrum of a sample of purified enzyme (1mg) in 50mM-potassium phosphate buffer, pH 7.5 (3.0ml), was recorded over the wavelength range 220–500nm against a buffer blank, in a Pye–Unicam SP.1800 recording spectrophotometer, with 1cm path-length cells.

Activation and inhibition studies

The effects of AMP (5mM), FAD (10μM), FMN (10μM) or riboflavin (10μM) on the activity of the purified NADH dehydrogenase were assessed by including these compounds in the standard assay solution without preincubation with the enzyme. EDTA, KCN and *p*-chloromercuribenzoate were preincubated (with the enzyme) in 0.2M-phosphate buffer, pH 7.5, for 30min at 23°C before samples were taken for assay.

Relationship between NADH dehydrogenase activities of supernatant and pellet fractions

Since NADH dehydrogenase activity was detected in both supernatant (63% of total units) and pellet (37% of total units) fractions after centrifugation of crude extract at 270000*g* for 60min at 4°C, the presence of at least two distinct NADH dehydrogenases was suggested. The following findings showed that the activities had rather similar

characteristics. Firstly, when samples of supernatant and pellet fractions were applied to, and subsequently eluted from, a hydroxyapatite column, the elution patterns of NADH dehydrogenase activity for both fractions were identical (Fig. 1). K_m values for NADH for both fractions were similar (approx. 100μM), and both supernatant and pellet activities showed substrate inhibition at concentrations of NADH greater that 700μM. Addition of AMP (5mM) to samples of both fractions elicited the same degree of inhibition of NADH dehydrogenase activity (approx. 20%). Using similar criteria, Dancey *et al.* (1976) were able to show a sharp distinction between the soluble and particulate NADH dehydrogenase activities in *Escherichia coli* extracts. In order to maximize yields of activity, it was decided to fractionate the whole extract from *B. stearothermophilus*, after solubilizing NADH dehydrogenase activity in the pellet (see below).

Solubilization of membrane-bound NADH dehydrogenase activity

Since about 37% of the NADH dehydrogenase activity was initially insoluble, i.e. was sedimented after centrifugation at 270000*g* for 1h, a method of solubilizing this particle-bound activity was devised. This consisted of addition of 2M-NaCl to the crude extract, followed by stirring for 1h, heating to 70°C for 1h, and finally centrifuging at 270000*g* for 1h. Treatment with 2M-NaCl at 23°C did not result in appreciable solubilization of NADH dehydrogenase activity.

Purification of NADH dehydrogenase activity

Step 1: treatment with 2M-NaCl at 70°C. The NADH dehydrogenase activity of *B. stearothermophilus* is appreciably heat-resistant: at 70°C in the presence of 2M-NaCl, considerable precipitation of inactive protein occurs (Mains, 1978). NaCl was added to crude extract (26.0ml) to give a final concentration of 2M. After stirring for 1h at 23°C, the extract was incubated at 70°C for a further 1h, cooled to 5°C, and the precipitated protein removed by centrifugation at 270000*g* for 1h at 8°C. The supernatant, containing approx. 71% of the initial total NADH dehydrogenase activity, was dialysed against several changes of 50mM-Tris/HCl, pH7.5 at 4°C. After dialysis to remove the 2M-NaCl, the NADH dehydrogenase activity remained soluble.

Step 2: chromatography on DE-52 anion exchanger. The dialysed supernatant (34.5ml) was applied to a column (15cm × 2.4cm) of Whatman DE-52 anion exchanger, pre-equilibrated with 50mM-Tris/HCl, pH7.5. Elution was performed at 23°C with a linear gradient of NaCl at a flow rate of 54ml/h. The gradient was prepared by mixing 50mM-Tris/HCl buffer, pH7.5, containing 500mM-NaCl (100ml) with 50mM-Tris/HCl buffer, pH7.5 (100ml). Eluate fractions (9.0ml) were assayed for protein and NADH dehydrogenase activity. The active fractions were pooled, and dialysed against several changes of 5mM-potassium phosphate buffer, pH6.8, at 4°C.

Step 3: chromatography on hydroxyapatite. The dialysed fractions (37.5ml) were next chromato-

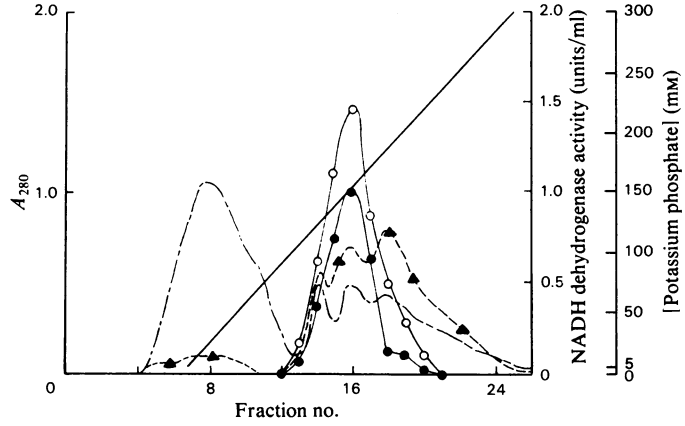

Fig. 1. *Chromatography of particle-bound and soluble NADH dehydrogenase on a column (15cm × 2.6cm) of hydroxyapatite*

The elution profiles of particle-bound and supernatant samples are superimposed on this Figure; however, they were run separately, with elution with a linear potassium phosphate gradient (——; 5–300mM, pH6.8, 150ml) at a flow rate of 32ml/h with collection of 8.0ml fractions. The protein (A_{280}) traces for the pellet fraction (▲) and supernatant fraction (-——-) are shown. NADH dehydrogenase activity in supernatant (○) and pellet (●) fractions was measured as described in the Materials and methods section.

graphed on an hydroxyapatite column (10cm × 2.4cm) which had been equilibrated with 5mM-potassium phosphate buffer, pH6.8. Elution was performed at 23°C with a gradient of potassium phosphate buffer prepared by mixing 50ml of 5mM-potassium phosphate buffer, pH6.8, with 50ml of 300mM-potassium phosphate buffer, pH6.8, at a flow rate of 30ml/h. Fractions (5ml) were assayed for protein and NADH dehydrogenase activity; the active fractions were pooled and dialysed against several changes of 50mM-potassium phosphate buffer, pH7.5, at 4°C.

Step 4: affinity chromatography on immobilized Cibacron Blue 3GA. The pooled, dialysed enzyme fractions (30ml) were applied to a column (15cm × 2.14cm) of immobilized Cibacron Blue 3GA which had been equilibrated with 50mM-potassium phosphate buffer, pH7.5. Elution (36ml/h) at 23°C with this buffer was continued until no more protein was detected in the eluate. NADH dehydrogenase was eluted with 50mM-potassium phosphate buffer, pH7.5, containing NADH (10mM). Active fractions were pooled and concentrated by ultrafiltration. NADH was removed from the resulting sample by gel filtration: the sample (4ml) was applied to a column (30cm × 1.5cm) of Sephadex G-50, and eluted with 50mM-potassium phosphate, pH7.5, at a flow rate of 16ml/h. The protein fractions (2.0ml/fraction) were pooled and concentrated by ultrafiltration.

Results

The results of the solubilization of NADH dehydrogenase from crude extracts of *B. stearothermophilus* are shown in Tables 1 and 2. The results of the purification are shown in Table 3 and Fig. 2. The enzyme was purified 104-fold and with an overall yield of 25%. The final gel filtration, conducted to remove excess NADH, resulted in some loss (approx. 30%) of NADH dehydrogenase activity. The heat-stability of the purified enzyme is indicated in Figs. 3 and 4; it retained a significant fraction of its activity after 10min at 70°C (Fig. 4).

The purified enzyme was relatively unstable on storage at −12°C; an activity loss of 75% occurred in 3 weeks. However, addition of NADH (1mM) to the solution improved the storage life, so that virtually no loss of activity occurred over a 4-week period.

Properties of the enzyme

Purity. Polyacrylamide-gel electrophoresis of the purified NADH dehydrogenase showed only one major band of protein, which coincided with the enzymic activity as assayed on gel sections (Fig. 5).

Molecular weight. Both polyacrylamide-gel electrophoresis in the presence of SDS and gel filtration on Sephadex G-200 indicated a mol.wt. of approx. 43000 for the purified enzyme (Mains, 1978). In addition, SDS/polyacrylamide-gel electrophoresis (Fig. 6) demonstrated the high degree of purity of the NADH dehydrogenase.

Absorption spectrum. The absorption spectrum of the purified enzyme (Fig. 7) showed peaks at 268 and 450nm and shoulders at 375 and 475nm. These spectral characteristics would, assuming a mol.wt. of

Table 2. *Behaviour of NADH dehydrogenase activity in soluble and particulate fractions, after treatment with 2M-NaCl and heat to 70°C for 1h*

The activity of NADH dehydrogenase in pellet and supernatant fractions, after initial centrifugation at 270000*g* for 1h, and separate treatment of both fractions with 2M-NaCl and heat to 70°C for 1h, was determined by the assay method described in the Materials and methods section.

	Total NADH dehydrogenase activity (units)	
	Supernatant	Pellet
Initial activity after centrifugation	39	23
Activity remaining after 2M-NaCl/heat treatment	28	19

Table 1. *Solubilization of NADH dehydrogenase from crude extracts of B. stearothermophilus*

The distribution of NADH dehydrogenase activity in pellet and supernatant fractions, after centrifugation either alone or after treatment with NaCl and heat, was determined by the assay method described in the Materials and methods section.

Method	Recovery of NADH dehydrogenase (%)	Percentage of recovered activity in supernatant	Percentage of recovered activity in pellet
Centrifugation of crude extract at 270000*g* for 1h	98	63	37
Addition of 2M-NaCl, heat to 70°C for 1h, and centrifugation at 270000*g* for 1h	75	94	6

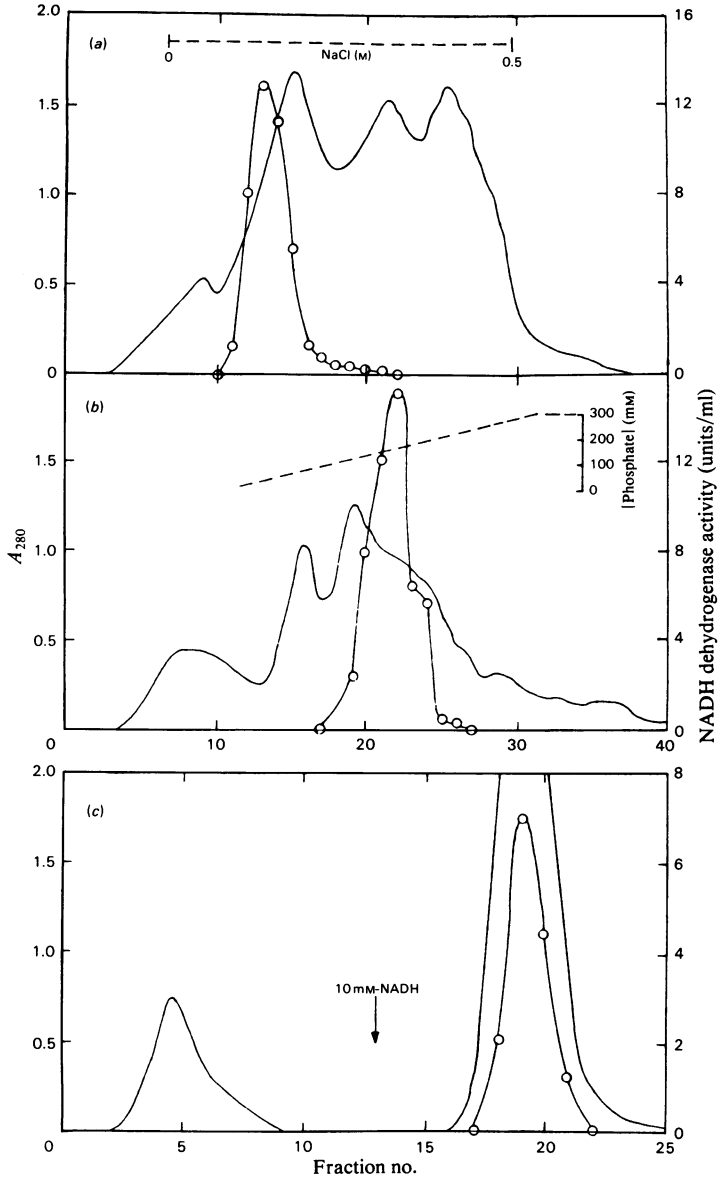

Fig. 2. *Chromatography of NADH dehydrogenase on DE-52 anion exchanger, hydroxyapatite and immobilized Cibacron Blue 3GA*

The details of the chromatography of NADH dehydrogenase on ion-exchange (*a*), hydroxyapatite (*b*) and affinity (*c*) columns are described in steps 2, 3 and 4 of the purification in the Materials and methods section. The protein trace (A_{280}, ——) and NADH dehydrogenase activity (O) for the collected fractions are shown. The enzyme activity was measured as detailed in the Materials and methods section.

43000, give ε_{450} for the enzyme of approx. $23.2 \times 10^3\,M^{-1}\cdot cm^{-1}$. Also, assuming ε for FMN or FAD to be $13.0 \times 10^3\,M^{-1}\cdot cm^{-1}$ (Brewer *et al.*, 1974), approx. two residues of flavin/molecule of protein are indicated.

Activators and inhibitors. AMP, FAD and riboflavin had no effect on the activity of the purified enzyme. However, FMN (10μM) produced an increase in enzyme activity up to 40% activation. This degree of activation was also observed at 0.5μM-FMN; indeed, no further activation was seen at FMN concentrations greater than 0.5μM. Varia-

Table 3. *Purification of NADH dehydrogenase from B. stearothermophilus*
The details of the purification procedures are given in the text and the legend to Fig. 2.

Purification step	Volume (ml)	Total protein (mg)	NADH dehydrogenase (units)	Specific activity (units/mg)	Purification factor (-fold)	Yield (%)
Crude extract	26.0	780.0	724.4	0.9	1.0	100.0
1. Supernatant (dialysed)	34.5	225.0	444.0	2.0	2.2	61.3
2. Ion-exchange column (dialysed active fractions)	37.5	55.0	290.0	5.3	5.8	40.0
3. Hydroxyapatite column (dialysed active fractions)	30.0	36.1	210.1	5.8	6.4	29.0
4. Active fractions from affinity column	48.0	1.9	178.5	94.0	104.4	24.6

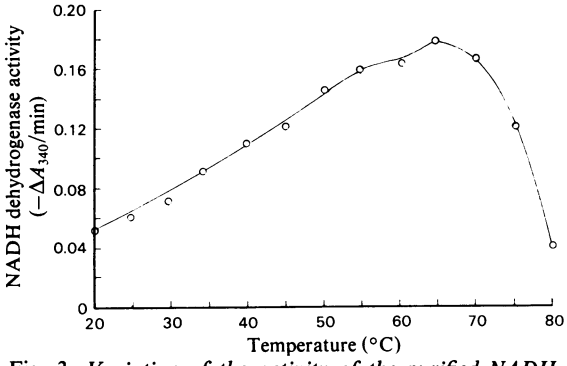

Fig. 3. *Variation of the activity of the purified NADH dehydrogenase with temperature*
The activity of the purified enzyme was monitored at reaction temperatures between 20 and 80°C. Assays were performed as described in the Materials and methods section with variation of the reaction temperature by means of a thermostatically controlled cell holder in the spectrophotometer.

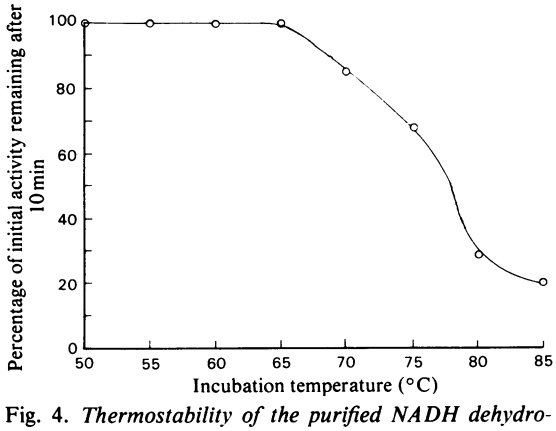

Fig. 4. *Thermostability of the purified NADH dehydrogenase*
The thermostability of the purified NADH dehydrogenase was ascertained by incubating samples (0.5 ml, 0.36 mg of protein) of purified enzyme preparation at various temperatures for 10 min, withdrawing a portion and assaying for NADH dehydrogenase activity as described in the Materials and methods section.

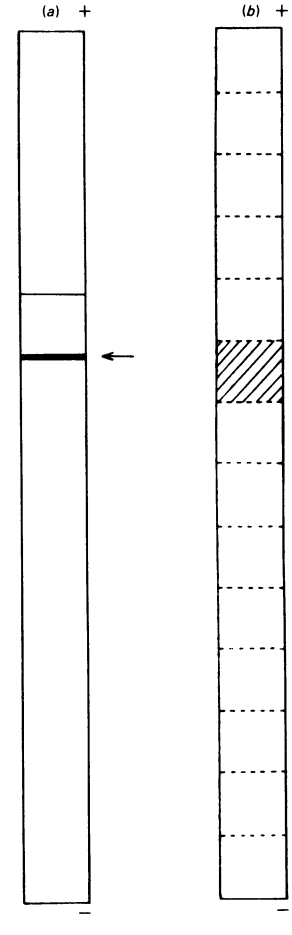

Fig. 5. *Polyacrylamide-gel electrophoresis of purified NADH dehydrogenase*
Polyacrylamide-gel electrophoresis in 7.5% acrylamide and 0.2% bisacrylamide was performed as described in the Materials and methods section. (*a*) Diagrammatic representation of the gel, with the NADH dehydrogenase identified by the arrow, and (*b*) the profile of located NADH dehydrogenase activity in sections of the gel, with the enzyme activity indicated by shading.

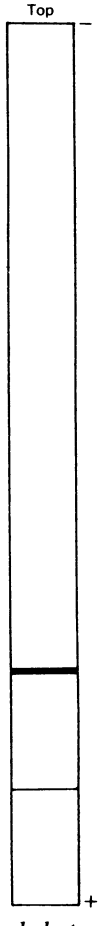

Fig. 6. *Polyacrylamide-gel electrophoresis of the purified enzyme in the presence of SDS*

Polyacrylamide-gel electrophoresis in the presence of SDS was performed in the Materials and methods section. The Figure shows a diagrammatic representation of the gel, stained for protein.

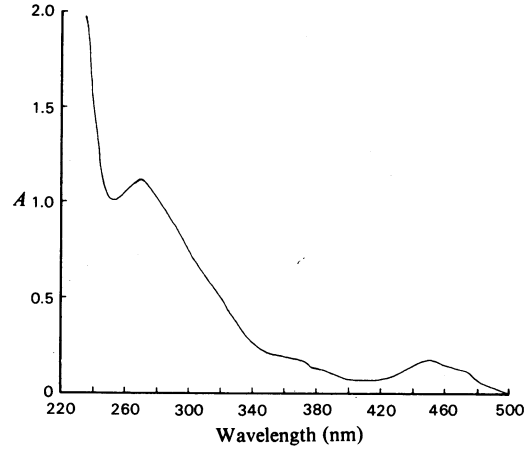

Fig. 7. *Absorption spectrum of the purified NADH dehydrogenase*

The absorption spectrum of the purified enzyme was measured as described in the Materials and methods section.

tion of enzyme activity with FMN concentration, when expressed as $1/v$ against the reciprocal of the FMN concentration, gave K_m for FMN of 0.025 μM.

Preincubation with KCN (50 mM) stimulated the activity of the purified enzyme by about 20%. Inhibition (50%) of the enzymic activity was observed on preincubation with *p*-chloromercuribenzoate (1.5 mM). No further concentrations of *p*-chloromercuribenzoate were tested for inhibition properties. Also, complete inhibition was achieved with EDTA (100 μM).

Discussion

The NADH oxidase system, including the NADH dehydrogenase, of the electron-transport chain of the thermophilic bacterium *B. stearothermophilus* has been shown to be membrane-bound (Wisdom & Welker, 1973). These workers found that sonication of cells of *B. stearothermophilus* followed by the addition of EDTA to the protoplasts revealed that most of the NADH oxidase was associated with the protoplast membrane. Our results show that approx. 40% of the NADH dehydrogenase activity in cell-free extracts is still membrane-bound, the activity in the high-speed supernatant being presumably due to (i) the method of cell breakage, causing solubilization of membrane-bound enzyme activity, and (ii) the presence of other NADH dehydrogenases. Treatment of crude extracts of *B. stearothermophilus* with 2 M-NaCl at 70°C causes almost complete solubilization of the membrane-bound activity (Table 1); virtually no activity is detected in the pellet fraction. This convenient solubilization procedure was accompanied by some loss (approx. 29%) of NADH dehydrogenase activity. Experiments involving NaCl and heat treatment of the separate supernatant and pellet fractions (Table 2) showed similar losses of activity for each of the two fractions. Selective heat-inactivation of the particulate activity can thus be ruled out. An improvement in the procedure would be to treat the pellet fraction only with NaCl and heat; this would avoid partial denaturation of the soluble activity and lead to greater overall yield. Solubilization of a membrane-bound NADH dehydrogenase of an extremely halophilic bacterium, with NaCl as the solubilizing agent, has been reported (Hochstein, 1975).

The key step in the present purification procedure is the use of affinity chromatography on immo-

bilized Cibacron Blue. Cibacron Blue has been suggested as a ligand specific for enzymes containing a super-secondary structure termed the dinucleotide fold or the NAD domain (Thompson & Stellwagen, 1976). About 60 different enzymes have been chromatographed on dye-bound Sepharose affinity columns. The NADH dehydrogenase of *B. stearothermophilus* bound completely to the immobilized Cibacron Blue affinity column, which might suggest the presence of a dinucleotide fold in the enzyme, although the exact mechanism of binding was not investigated. The NADH dehydrogenase of *B. stearothermophilus*, when bound to the affinity column, was not eluted by inclusion of 2M-NaCl in the eluting buffer or by changing the pH of the buffer. Attempts to desorb the enzyme electrophoretically were unsuccessful, unlike the work of Dean *et al.* (1977), which reported the electrophoretic desorption of several dehydrogenases from immobilized-dye affinity columns. The specific ligand, NADH, proved successful at eluting the enzyme and for this reason no other was used. The exact mechanism of binding to, and eluting with substrate from, Cibacron Blue 3GA remains a matter for conjecture.

Electrophoresis on polyacrylamide gels with (Fig. 6) and without (Fig. 5) SDS showed only one major protein band with a minor contaminant. The extent of purification of the NADH dehydrogenase of *B. stearothermophilus* compared favourably with respiratory-chain-linked NADH dehydrogenases isolated and purified from other bacterial sources. Indeed, according to Jinks & Matz (1976), purification factors of bacterial NADH dehydrogenases of 20–30-fold and yields of 10–30% indicate a preparation of about 90% purity.

The observation that the purified enzyme and maximum stability when stored in the presence of its substrate, NADH, agrees with the observations of Hochstein & Dalton (1973), who found that the stability of the respiratory-chain-linked NADH dehydrogenase from a halophilic bacterium was dramatically increased in the presence of NADH. Such substrate stabilization has also been observed with other bacterial enzymes (Hochstein & Dalton, 1973). The remarkable thermostability of the purified enzyme (Fig. 4) is in marked contrast with that of the NADH dehydrogenase from mammalian mitochondria, which has been shown to lose 90% of its activity on heating to 60°C for 10min (Mains, 1978), and the enzyme from *Bacillus cereus*, which loses all activity on heating to 70°C for 10min (Amelunxen & Lins, 1968).

The absorption spectrum of the purified NADH dehydrogenase is indicative of a flavoprotein, as shown by the similarity of its absorption maxima to those of well-characterized flavoproteins (Swoboda & Massey, 1965; Massey & Williams, 1965; Knight *et al.*, 1966; Palmer & Massey, 1968; Hatefi & Stigall, 1976). No fluorescence studies were performed to support this hypothesis.

FMN stimulated the activity of the purified enzyme. This indicates that in the purified form the NADH dehydrogenase had a specific, high affinity for FMN, since neither FAD nor riboflavin had any effect on the enzyme activity. This requirement for a specific flavin to stimulate activity has also been shown for the NADH dehydrogenase from *E. coli* (Dancey & Shapiro, 1976), where FAD was used. The prosthetic group of the NADH dehydrogenase of *B. stearothermophilus* is therefore most probably FMN. Removal of this possibly loosely bound moiety may be an explanation for the decrease in activity of the enzyme after gel filtration. For this reason, the flavin stoichiometry of 2 (calculated from the A_{450} of the purified enzyme) must be regarded as an approximation. Also, at this stage, an iron–sulphur contribution to the 450nm band cannot be precluded. Alternatively, inactivation on gel filtration may be due to the oxidation of thiol groups, the presence of which is implied by inhibition of enzymic activity with *p*-chloromercuribenzoate. No attempt was made to reverse the suggested oxidation of thiol groups by using mercaptoethanol or dithiothreitol, since both these reagents give appreciable non-enzymic reductions of dichlorophenol-indophenol, thereby interfering with the enzyme assay.

The inhibition of activity by EDTA may possibly indicate that metal ions are essential for the activity of the enzyme.

The increase in enzyme activity after addition of KCN has also been shown for the NADH dehydrogenase of *E. coli* (Dancey & Shapiro, 1976), where KCN (5–10mM) was reported to enhance the activity by almost 100%. In this latter case it has been suggested that KCN has a direct effect on the interaction of the electron acceptor, dichlorophenol-indophenol, with the enzyme. Alternatively, this increase in enzyme activity described for both *E. coli* and *B. stearothermophilus* may involve repair of thiol groups essential for activity (Lin *et al.*, 1975).

I. M. was the recipient of a studentship from the Science Research Council.

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