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PURIFICATION OF MITOCHONDRIAL NADH DEHYDROGENASE FROM *DROSOPHILA HYDEI* AND COMPARISON WITH THE 'HEAT-SHOCK' POLYPEPTIDES

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Summary

Mitochondrial NADH dehydrogenase (NADH:(acceptor) oxidoreductase, EC 2.6.99.3) from either *Drosophila hydei* larvae or embryos has been purified 150- and 120-fold, respectively. The purified enzyme appeared homogeneous and showed a molecular weight of 57 000. The molecular weight of the non-denatured enzyme was 79 000. On isoelectro-focussing of the preparation, two fractions were observed, a major one with an isoelectric point of 6.2 and a minor fraction with an isoelectric point of 4.9.

Straight-line kinetics in Lineweaver-Burk plots were observed for the purified enzyme with a K_m of 0.040 mM. The K_m was not changed during the purification procedure, suggesting that the enzyme was not denatured or inactivated. The pH optimum of the purified enzyme was 5.6.

The molecular weight of the purified mitochondrial NADH dehydrogenase does not correspond to that of one of the 'heat-shock' polypeptides.

Introduction

Treatments, such as heat-shock or interference with the cellular respiratory metabolism, result in the induction of specific puffs in the polytene chromosomes of *Drosophila* larvae [1—4]. During these treatments, six new labelled bands (the 'heat-shock' bands) appear in the protein synthetic pattern of salivary glands of *D. hydei* [5—7]. Furthermore, during these treatments the activity of a variety of mitochondrial enzymes increases e.g. after CO_2

Abbreviation: MES, 2-(*N*-morpholino)ethanesulfonic acid.

anaerobosis of salivary glands, the apparent V of NADH dehydrogenase (NADH:(acceptor) oxidoreductase, EC 1.6.99.3) reached a value of 140% of the control value [8]. Koninkx [9] found a correlation between the increase in NADH dehydrogenase activity and the activity of puff 4-81 B (one of the 'heat-shock' puffs). Electrophoresis of mitochondrial preparations of glands treated for puff induction showed a significantly higher incorporation of [^{35}S]-methionine in fractions with NADH dehydrogenase activity as compared to control glands. These experiments suggested the possibility that the de novo synthesized NADH dehydrogenase could be one of the 'heat-shock' bands. We have therefore purified this enzyme, according to the procedures described below, from both *D. hydei* larvae and embryos and have determined its molecular weight. Also several of its physical properties are described.

Materials and Methods

Materials. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from B.D.H. Chemicals, Poole, Dorset, U.K.; *N,N,N',N'*-tetramethylethylendiamine, sodium dodecyl sulphate (SDS) and Triton X-100 were from Serva, Heidelberg, Germany; β-NADH (disodium salt; grade 11) and standard proteins for molecular weight estimation from Boehringer GmbH, Mannheim, Germany; β-mercaptoethanol and 2-(*N*-morpholino)ethanesulfonic acid (MES) from Sigma Chemical Co., St. Louis, U.S.A.; ampholine was from L.K.B., Bromma, Sweden; DEAE-cellulose (DE-52) was from Whatman, Maidstone, England; [^{35}S]methionine (specific activity 300 Ci/mmol) was from The Radiochemical Centre, Amersham, U.K.; CNBr-activated Sepharose 4B and Sephacryl S-200 Superfine were from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade from Merck AG, Darmstadt, Germany.

Enzyme assay. Mitochondrial NADH dehydrogenase activity was assayed by following the rate of NADH oxidation spectrophotometrically at 340 nm: 10 µl enzyme solution was added to 330 µl 0.2 N sodium acetate buffer (pH 5.6)/0.1% Triton X-100/0.3 mM $K_3Fe(CN)_6$.

The reaction was started by addition of 6 µl 5 mM NADH. Enzyme activity is expressed in units (1 unit = 1 µmol NADH consumed/min per µg protein), using a molar absorption coefficient for NADH of 6.22.

The assay is not affected by lipoamide dehydrogenase activity, since it is not inhibited by arsenite.

Protein determination. To prevent Triton X-100 precipitation upon addition of the Folin reagent, protein was determined according a modified Lowry procedure in the presence of SDS [10].

Isolation and labelling of salivary glands. Hand-isolated salivary glands from late-third-instar larvae of *D. hydei* were isolated and labelled as described by Koninkx [6].

Preparation of mitochondria. Mitochondria were prepared from two different sources. Firstly from 10—15-h-old embryos of a wild type stock of *D. hydei*. The embryos were dechorionated in water/ethanol/chlorox (1 : 2 : 1, v/v) for 2 times 1 min and homogenized in a glass-Teflon homogenizer in 10 vols. of ice-cold 0.154 M KCl/1 mM EDTA (pH 7.2). The homogenate was clarified twice at 1000 × *g* (Sorval HB-4 rotor) for 5 min. Mitochondria were

then collected from the supernatant solution by centrifugation at 6000 $\times g$ for 10 min. The pellet was resuspended in homogenization buffer, recentrifuged at 6000 $\times g$ for 10 min and stored at −30°C until use. Secondly, late-third-instar larvae of the same *D. hydei* stock were used. The method of Boyd et al. [11] for the mass preparation of larval salivary glands was followed, except for the fact that larval gut and Malpighian tubules were not removed. Yeast was removed by washing the suspension on a very fine mesh gauze with Mg^{2+}-Ringer [12] until the wash solution was clear. The suspension was collected by centrifugation at 3000 rev./min for 1 min. Mitochondria were prepared as described above for embryos.

Sephacryl S-200 superfine gel filtration. Gel filtration was carried out in a (35 × 1.6 cm) Sephacryl S-200 superfine column, packed at a flow rate of 78 ml/h equilibrated in buffer 1 (0.01 M Tris/MES)0.02% Triton X-100, pH 6.8). Because of the absorption of Blue Dextra at pH below 7 at low ionic strength (Pharmacia Fine Chemicals information) and the interactions between Blue Dextran and NADH dehydrogenase, the void volume was determined with ferritin. This protein has a molecular weight of 450 000, which is well above the exclusion limit of Sephacryl S-200 superfine.

Ion-exchange chromatography. For ion-exchange chromatography a (5 × 1.6 cm) column of DEAE-cellulose (DE-52) was used equilibrated in buffer 1.

Preparation of the Blue Dextran-Sepharose 4B absorbent. 5 g CNBr-activated Sepharose 4B was pretreated with 1000 ml 1 mM HCl as recommended by the manufacturer. 0.25 g Blue Dextran was dissolved in 0.1 M $NaHCO_3$ and added to the gel. The suspension was then gently mixed at room temperature for 2 h. Unbound dye was removed by several changes of buffer. To block remaining reactive groups the gel suspension was treated with 1 M triethanolamine at pH 8.0 for 2 h at room temperature and subsequently equilibrated in buffer 1. The gel was stored at 4°C in the presence of 0.02% NaN_3. For affinity chromatography, the gel was used as a 6 × 1.6 cm column equilibrated in buffer 1.

Polyacrylamide gel electrophoresis. Electrophoresis in 10% polyacrylamide gels in a discontinuous urea/SDS system and preparation of the samples was performed as described by Koninkx [6]. When Triton X-100 was present in the samples, SDS was added to a concentration equivalent to that of the Triton X-100 before the trichloroacetic acid precipitation [13].

Molecular weight estimation. Molecular weight was determined by gel filtration of the purified enzyme on a Sephacryl S-200 superfine column.

The column was equilibrated with 0.1 M Tris-HCl/200 mM NaCl (pH 7.5). Calibration of the column was performed with aldolase (158 000), albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 500).

Polyacrylamide gel isoelectrofocussing. Polyacrylamide gel isoelectrofocussing was performed using Ampholine polyacrylamide gel plates (pH range 3.5—9.5) on a LKB 2117 Multiphor.

Voltage was held constant at 150 V for 24 h at 10°C. After focussing, the gel was stained for enzyme activity by incubation at 4°C in 0.1 M Tris-HCl (pH 7.2)/0.5 mM NADH/0.5 mM oxidized 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide. The gel was then sliced an the slices were shaken in H_2O for 2 h. The pH of the eluate was then determined.

Determination of the kinetic constants. Apparent K_m and V values were calculated from the intercept values of Lineweaver-Burk plots. In these plots, the inverse NADH concentration was plotted against the inverse NADH units oxidized/mg protein. 9 different NADH concentrations were used ranging from 0.028 to 0.28 mM. The assay conditions used were the same as described above except for NADH concentrations.

Results

Purification of the mitochondrial NADH dehydrogenase

All purification procedures were carried out at 0—4°C unless indicated otherwise.

Mitochondrial NADH dehydrogenase is known to be a membrane-bound enzyme, but can be solubilized from a variety of tissues by extraction with Triton X-100. This is also the case in *D. hydei*: when the mitochondrial pellet was homogenized in 0.01 M Tris/MES (pH 6.8) and adjusted to 0.5% Triton X-X-100 and left stirring at room temperature for 2 h, a major part of the enzyme activity was solubilized. Following cooling to 2°C and clarification at 100 000 $\times g$ for 1 h, the extract could be used as starting material for the purification of the enzyme. The extract obtained was not clear due to a small amount of lipid. This material was eliminated in the subsequent gel filtration step.

When the Triton extract was applied to a Sephacryl S-200 superfine column, most of the protein material (as measured by absorbance at 280 nm) eluted in the void volume of the column, while the NADH dehydrogenase activity eluted just after the main protein peak (Fig. 1).

Fractions containing more then 5 units NADH dehydrogenase were pooled. The pooled fractions of the extract contained 21% of the total protein and 82% of the NADH dehydrogenase activity. Using the embryo extract 23% of the total protein was pooled containing 78% of enzyme activity.

The crude NADH dehydrogenase obtained after gel filtration was further purified by ion-exchange chromatography on DE-52 cellulose. Most of the con-

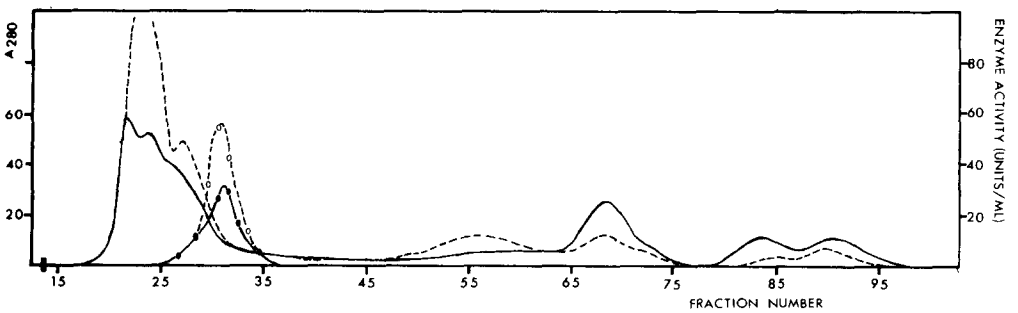

Fig. 1. Elution profile of crude mitochondrial proteins from a Sephacryl S-200 superfine column (35 × 1.6 cm). The column was equilibrated and eluted with 0.01 M Tris/MES (pH 6.8)/0.02% Triton X-100. 4 ml Triton X-100 extract was applied to the column (1.4-ml fractions, 10 ml/h) (———) and (- - - - -) A_{280} absorbance of proteins; o———o, and o- - - - -o, NADH dehydrogenase activity from *D. hydei* larvae and embryos, respectively.

taminating proteins are not bound to the column at low ionic strength (0.01 M Tris/MES) and could be washed through with the starting buffer. A linear NaCl gradient was then applied to the column to elute the NADH dehydrogenase. The peak of enzyme activity was found at 0.2 M NaCl (Fig. 2). Again. fractions containing more than 5 units of enzyme activity were pooled and concentrated by ultrafiltration under N_2 pressure through an Amicon PM-10 membrane.

During this purification step about 45% of both the larval and embryonic NADH activity is lost, presumably due to irreversible adsorption to the ion-exchange column and non-specific adsorption by the ultrafiltration membrane. The remainder contained 7% protein of the larval extract with 46% of the enzyme activity or 5.5% of the protein of the embryo extract with 44% of the enzymatic activity.

This material was then reapplied to the Sephacryl S-200 superfine column. The column was developed under the sane conditions as before and yielded a further 4-fold purification.

As the last step in the purification scheme, the pooled fractions from the second gel filtration step were applied to a Blue Dextran-Sepharose 4B column. NADH dehydrogenase bound tightly to this column, while most of the contaminant proteins did not and could be washed off with the equilibration buffer.

The elution of NADH dehydrogenase could then be achieved by raising both the ionic strength to 0.01 M Tris-HCl/0.5 M NaCl/0.02% Triton X-100 and the pH to 7.2 (Fig. 3).

Fractions with enzyme activity were pooled and concentrated in a Minicon B15 cell. The resulting material was judged to be essentially pure by SDS-polyacrylamide gel electrophoresis.

The purification scheme is summarized in Table I for *D. hydei* larvae and

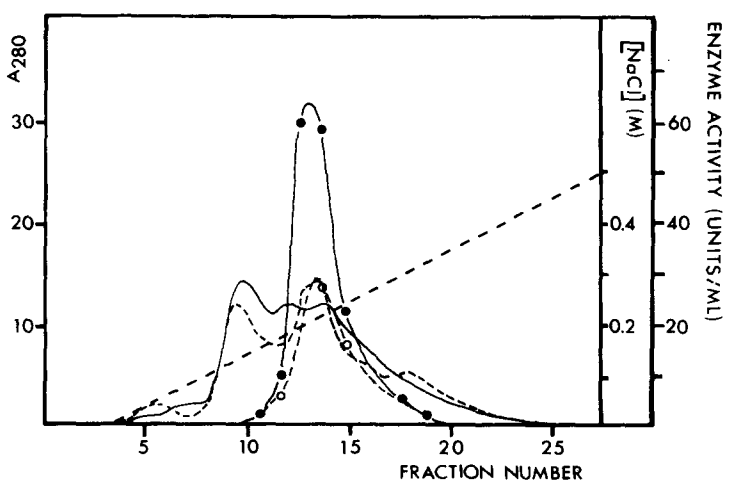

Fig. 2. Elution profile of proteins from a DE-52 cellulose column (5 × 1.6 cm, 2-ml fractions, flow rate, 20 ml/h). The column was equilibrated with 0.01 M Tris/MES (pH 6.8)/0.02% Triton X-100 and eluted with a linear NaCl gradient (0—0.5 M). ———, and - - - - - -, A_{280} absorbance of proteins; o———o, and o- - - - -o, NADH dehydrogenase activity from *D. hydei* larvae and embryos, respectively.

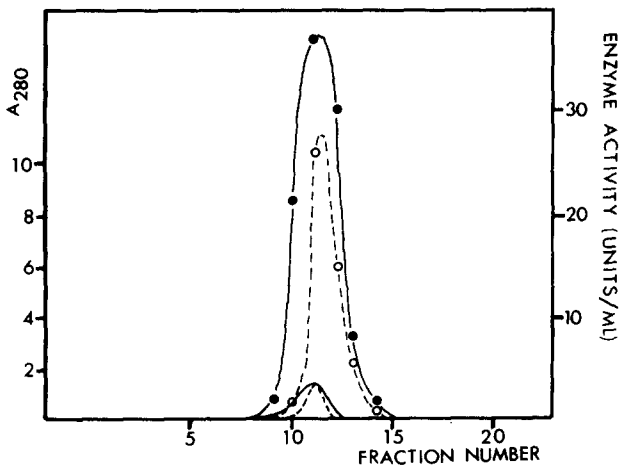

Fig. 3. Elution profile of proteins from a Blue Dextran-Sepharose 4B column (6 × 1.6 cm, 2 ml fractions, 20 ml/h). The column was equilibrated with 0.01 M Tris/MES (pH 6.8)/0.02% Triton X-100 and eluted with 0.5 M NaCl in the same buffer. ———, and - - - - - -, A_{280} absorbance of proteins; o———o, and o- - - - - -o, NADH dehydrogenase activity from *D. hydei* larvae and embryos, respectively.

embryos. A purification of 120-fold was achieved with a recovery of 27% for embrhonic NADH dehydrogenase and 151-fold with a recovery of 30% for the larval enzyme.

Properties of the purified enzyme

The following holds for both enzymes, since their characteristics appeared to be the same.

Molecular weight estimation. Molecular weight of the purified NADH dehydrogenase was determined by gel filtration on a Sephacryl S-200 superfine column, calibrated with proteins of known molecular weight the molecular weight was calculated as 79 000.

Subunit molecular weight estimation. The purified NADH dehydrogenase

TABLE I

SUMMARY OF ENZYME PURIFICATION

Results are for 100-g damp *D. hydei* larvae and 14-g dechorionated embryos. L, larval extract; E, embryo extract.

Purification step	Enzyme activity (units)		Protein (mg)		Spec. activity (units/mg)		Yield (%)		Purification (-fold)	
	L	E	L	E	L	E	L	E	L	E
Triton X-100 extract	446	286	14.00	15.20	32	19	100	100	1	1
Sephacryl S-200 superfine gel filtration	366	333	3.95	3.48	124	64	82	78	4	3
DE-52 cellulose chromatography	206	125	0.97	0.83	214	151	46	44	7	8
Sephacryl S-200 superfine filtration	165	107	0.17	0.23	948	477	37	37	30	25
Blue Dextran-Sepharose 4B affinity chromatography	135	76	0.03	0.11	4822	2260	30	27	151	120

was subjected to electrophoresis in 10% polyacrylamide gel containing urea and SDS. The NADH dehydrogenase migrated as a single protein band. The subunit size was estimated to be 57 000.

Isoelectrofocussing. The purified enzyme was further analyzed by isoelectrofocussing in polyacrylamide gel electrophoresis. After focussing the gel was stained both for enzyme activity and for protein. Two fractions with enzymatic activity were observed: one major and one minor band with isoelectric points of 6.2 and 4.9, respectively. Only the major band could be stained with Coomassie Blue.

Effect of pH. The purified enzyme exhibited a pH optimum of 5.6 when assayed with NADH as substrate and $K_3Fe(CN)_6$ as electron acceptor.

Enzyme kinetics. The Lineweaver-Burk plots of the NADH dehydrogenase activity were linear between NADH concentrations of 0.028 and 0.083 mM (Fig. 4). The line was determined by the least squares method. The apparent K_m value for the purified enzyme (0.040 mM) does not differ significantly from that for the crude extract (0.041 mM). This indicates that the affinity for the substrate is not changed during the purification procedures. The apparent V value of the purified enzyme (7942 U/mg protein) has been increased 3900-fold compared to that of crude extract (20 U/mg protein).

Comparison of the 'heat-shock' polypeptides with the purified NADH dehydrogenase. To establish whether one of the 'heat-shock' polypeptides is part of the NADH dehydrogenase, the purified enzyme was subjected to

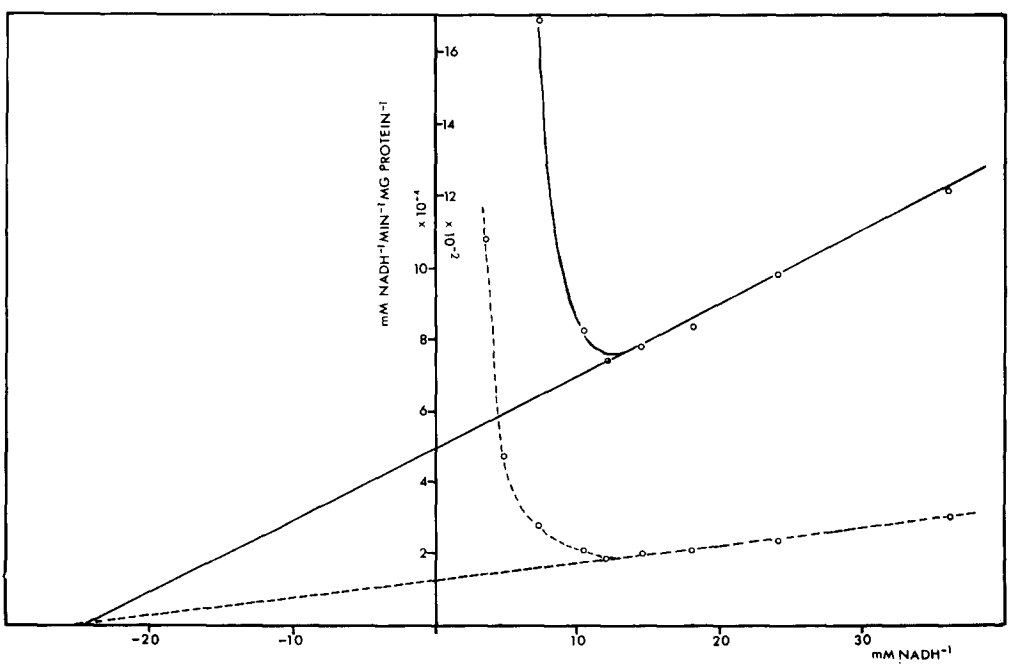

Fig. 4. Lineweaver-Burk plots of the purified NADH dehydrogenase (o- - - - -o) and the enzyme of the crude extract (o————o). The function was determined at 0.867 mM Fe $(CN)_6^{3-}$ and at varying NADH concentrations. The straight line was determined by the least squares method.

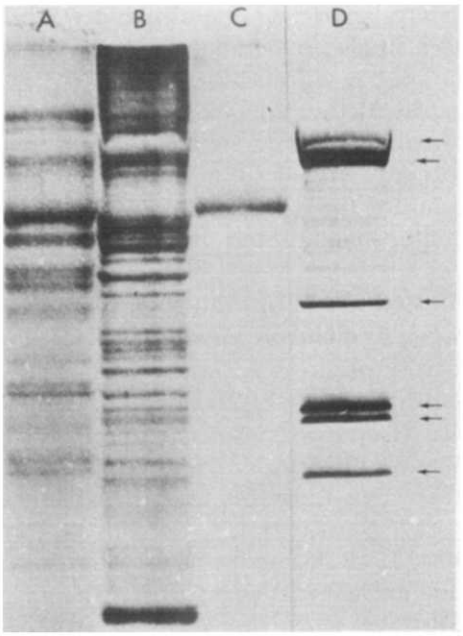

Fig. 5. SDS-polyacrylamide gel electrophoresis of mitochondrial NADH dehydrogenase from *D. hydei* before and after purification and salivary gland proteins extracted after temperature shock of 60 min at 37°C, labelled with [^{35}S]methionine. (A) crude Triton X-100 extract; (B) salivary gland extract; (C) purified enzyme; (D) autoradiography of B. (A), (B) and (C) were stained with Coomassie Blue. The arrows indicate the six 'heat-shock' polypeptides.

electrophoresis in 10% polyacrylamide gel containing urea and SDS. A salivary gland extract labelled with [^{35}S]methionine after a 60 min temperature shock at 37°C was applied to this gel. From the positions of the 'heat shock' polypeptides, visible after autoradiography, it is clear that the molecular weight of these polypeptides differs significantly from that of NADH dehydrogenase (Fig. 5).

Discussion

For solubilization of the enzyme, the non-ionic detergent, Triton X-100, was used. This detergent does not usually denature proteins and has been used by others to solubilize membrane-bound enzymes. NADH dehydrogenase was purified from a Triton extract by standard methods: gel filtration, ion-exchange chromatography and affinity chromatography. The elution of the dehydrogenase from the latter column may, however, be non-specific, since it could only be achieved with 0.5 M NaCl but not with NADH at concentrations up to 5 mM. This may indicate that the enzyme is not bound to the column by means of the NADH binding site. During this purification procedure the substrate (NADH) affinity of the mitochondrial NADH dehydrogenase was not changed suggesting that no major change has occurred in the enzyme. We estimate the molecular weight of the native enzyme to be 79 000 (by gel filtration) while the denatured enzyme, during SDS-polyacrylamide gel electro-

phoresis, contained only one polypeptide chain with a molecular weight of 59 000. The difference between the molecular weight as estimated by gel filtration and estimated by urea/SDS gel electrophoresis is significant, but is less than a factor of two. Thus the enzyme is probably a monomer in the native state, but may be still complexed with some lipids, thus increasing its molecular weight as compared to that found after denaturation and delipidation by SDS.

From bovine heart, several preparations of NADH dehydrogenase have been described. All can be reduced to two different types, namely a high molecular weight type (550 000) and a low molecular weight type (80 000) [14]. The low molecular weight type, however, seems to be a degradation product of the high molecular weight type, since the former fractions were isolated during procedures which involved exposure to heat, acid pH and ethanol or combinations thereof. The high molecular weight type was converted to the low molecular weight type by these treatments. The optimum pH for activity for these preparations was 8.5 [14,15]. From preparations of yeast electron-transport particle a NADH dehydrogenase has been isolated which differs in several respects from the enzyme prepared from bovine heart [16].

The molecular weight of our enzyme preparation was estimated to be in the range of 51 000—58 000 and the optimum pH for activity was 5.5. The enzyme isolated from *D. hydei* differs clearly from the bovine heart enzyme both in molecular weight and pH optimum of ferricyanide, but seems to be similar to that extracted from yeast. The molecular weight of the yeast enzyme (extracted with 9% ethanol at acidic pH) is close to that of the denatured *D. hydei* enzyme and both have the same pH optimum for ferricyanide.

Many attempts have been made to correlate the increase in activity of mitochondrial NADH dehydrogenase after experimental gene induction with six de novo synthesized peptides, which were also found after that induction. Most of the arguments to support such a correlation were, however, indirect [8,9,17]. More direct was the indication of Koninkx et al. [17] that the labelled mitochondrial protein fractions with NADH dehydrogenase activity migrated in urea/SDS-polyacrylamide gels in the range of proteins with molecular weights of 67 000—75 000, the range in which the first and second 'heat-shock' polypeptides migrated [6]. This report, however, shows that none of the 'heat-shock' peptides corresponds to NADH dehydrogenase since their molecular weights differ clearly. This difference is not due to a modification of the enzyme after gene induction treatments because the enzyme isolated from tissues subjected to CO_2 anaerobiosis has the same properties as the dehydrogenase isolated from non-treated tissues.

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