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A simple method for the purification of the mitochondrial NADH dehydrogenase

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SUMMARY

A simple procedure for the isolation and purification of the high-molecular-weight type NADH dehydrogenase from beef heart mitochondria is reported.

The soluble high-molecular-weight type NADH dehydrogenase[1] exemplified by Singer's enzyme[2], is believed to be a complex of a flavoprotein and an iron–sulfur protein (also known as the non-heme iron protein). A simple procedure for isolating the high-molecular-weight type NADH dehydrogenase, based on the observations of Cunningham *et al.*[3] and Kaniuga and Gardas[4], has been developed and is reported here. The enzyme preparation is stable during the prolonged storage. The isolation procedure involved the extraction of the lipid-deficient beef heart submitochondrial particles with a non-ionic detergent, and purification by means of an ion exchanger and calcium phosphate gel.

Submitochondrial particles were prepared by disruption of beef heart mitochondria in a sonic oscillator by the method of Pharo *et al.*[5] and were stored in 0.25 M sucrose at -20° until used. All steps involved in the isolation procedure were carried out at 0 to 3°. The thawed submitochondrial particles (8 g protein) after separation from the sucrose solution by centrifugation at 105 000 × *g* for 30 min, were suspended in 150 ml of a 50 mM phosphate buffer, containing 1 mM EDTA, pH 7.4 (phosphate buffer). The suspension was then shaken vigorously for 15 min with 900 ml of diethyl ether in a separatory funnel[4]. The ether in the aqueous emulsion was first removed by decantation after centrifugation at 1000 × *g* for 10 min and finally by suction for 15 min with an aspirator. The lipid-deficient particles were washed and subsequently resuspended in the phosphate buffer to a protein concentration of 10 mg/ml. After lowering the pH of the

Abbreviation: PCMS, *p*-chloromercuriphenylsulfonate.

suspension with 1 M acetic acid to 6.6, purified Lubrol WX powder* was added to a final concentration of 1%, and the mixture was stirred for 1 h. The mixture was centrifuged at 105 000 × *g* for 1 h and the pH of the yellow supernatant was readjusted to 7.4 with 1 M NH_4OH. The protein in the crude extract, collected after $(NH_4)_2SO_4$ precipitation (0.5 g of solid $(NH_4)_2SO_4$ per ml of extract, NH_4OH being added to maintain neutrality) and centrifugation, was desalted on a Sephadex G-25 column pre-equilibrated with a medium which was 0.1% in Lubrol, 1 mM in EDTA and 10 mM in Tris–sulfate, pH 7.4 (Tris buffer). The desalted protein solution was then applied to an anionic exchange column, DEAE-cellulose (microgranular form, DE-52, Whatman) and the column was eluted sequentially with Tris buffers containing 0, 25, 50, 75, 100 and 1000 mM KCl. All fractions, except for the first, showed substantial activity in ferricyanide reduction. However, the protein fraction which was eluted with buffer containing 50 mM KCl, had the highest specific activity and by far the strongest $g = 1.94$ electron paramagnetic resonance (EPR) signal[6] on reduction and was used for further studies. The DEAE-cellulose purified enzyme can be further purified by means of calcium phosphate gel[7] fractionation. The most active protein fraction (80 ml, protein concentration, 1 mg/ml) was first stirred with 80 mg of calcium phosphate gel for 5 min. The gel, which adsorbed approximately 20 mg of less pure protein, was subsequently removed by brief centrifugation at 1000 × *g*. A second aliquot of gel (120 mg) was used to adsorb the bulk of protein (40 mg) by the same procedures. The less pure protein adsorbed on the second aliquot of gel was removed by homogenizing the gel with 2.0 ml of 50 mM phosphate buffer, pH 7.4, containing 0.1% Lubrol, followed by centrifugation. Subsequent extraction of the gel with 1.2 ml of 100 mM phosphate buffer, pH 7.4, containing 0.1% Lubrol, yielded a protein fraction which had a very high specific activity. The enzyme preparation may be stored at −20° for several weeks without loss of activity. The yield, activity and purity of NADH dehydrogenase were summarized in Table I

The presence of detergent is presumably essential for the solubility of the enzyme. Attempts to remove Lubrol from the NADH dehydrogenase preparation by dialysis were unsuccessful, since Lubrol does not diffuse through the dialysis membrane at an appreciable rate. However, the detergent in the NADH dehydrogenase preparation extracted with Triton X-100, another non-ionic detergent, can be readily removed by extraction with diethyl ether. The enzyme became insoluble after the removal of the detergent but retained its reactivity (unpublished results).

The absolute absorption spectrum and the difference spectrum (reduced–oxidized) of NADH dehydrogenase are shown in Fig. 1. The difference spectrum, which was obtained by reduction with NADH, was very similar to that obtained with Singer's enzyme[9]. The trough at around 410 nm and the negative shoulder around 450 nm were probably due to the iron–sulfur and FMN prosthetic groups, respectively. The FAD in the preparation (Table I) may be associated with contaminating flavoprotein since the FMN content increased while FAD content decreased during purification.

*Lubrol WX, a generous gift from ICI America, Inc., Stamford, Conn., was purified as follows: The detergent (60 g) was dissolved in 120 ml of boiling ether–ethanol (5:1, v/v) containing 6 g of charcoal. The mixture was filtered through a fritted glass funnel (medium pore size) while warm. The white precipitate, which was formed when the filtrate was allowed to cool down slowly to 0°, was collected by filtration and was dried in a vacuum desiccator before use.

TABLE I

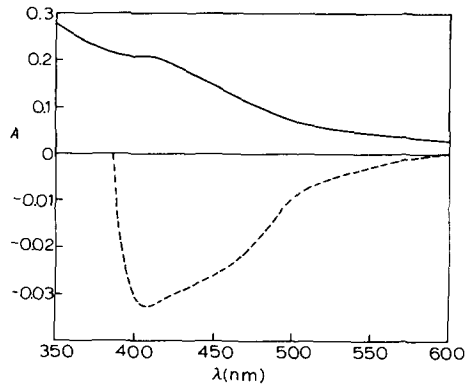
NADH DEHYDROGENASE PREPARATION

Protein	*Total protein (mg)*	*Specific activity*★		*FMN*★★ *(nmoles/mg)*	*FAD*★★ *(nmoles/mg)*	*Iron*★★★ *(ngatoms/mg)*
		At fixed acceptor concn.	V_∞			
Submitochondrial particles	8000	5.0	–	–	–	–
Crude extract	850	58	–	–	–	–
After DE-52 chromatography	80	90	–	0.84	0.18	26.9
After calcium phosphate gel fractionation	23	152	860	1.17	0.07	–

★The specific activities were determined at 30° with ferricyanide as acceptor, in 40 mM triethanolamine–HCl, pH 7.8, at a fixed ferricyanide concentration of 1.67 mM, and at several acceptor concentration levels for V_∞ according to Minakami *et al.*[8]. They were expressed as μmoles of NADH oxidized per min per mg of protein.

★★FMN and FAD were acid-extractable and determined by fluorimetric measurements as described by Udenfriend[10].

★★★Non-heme iron content was determined according to Doeg and Ziegler[11].



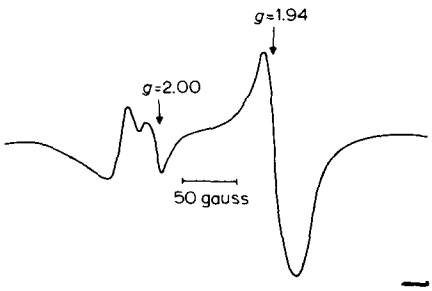


Fig. 1. Absorption spectra of NADH dehydrogenase. The enzyme preparation was in a medium which was 0.1% in Lubrol, 1 mM in EDTA, 10 mM in Tris–sulfate, pH 7.4, at a protein concentration of 1.2 mg/ml. The spectra were recorded on a Cary spectrophotometer, Model 15. ——, absolute absorption spectrum (oxidized form); - - - - -, reduced *minus* oxidized, obtained by addition of 5 μl of 50 mM NADH to 3 ml of enzyme solution in sample cuvette.

Fig. 2. EPR spectrum (first derivative) of 2.9 mg NADH dehydrogenase in 0.2 ml Tris buffer, reduced with 10 μl of 0.1 M NADH, frozen after 30 sec at 0°. Spectrum was recorded at 77°K.

The acceptor specificity and inhibitor sensitivities of the NADH dehydrogenase are typical of the soluble high-molecular-weight type enzyme[12]. For example, it exhibits high activity with ferricyanide and negligible activity towards ubiquinones, menadione, cytochrome *c*, and 2,6-dichlorophenolindophenol. Its ferricyanide reduction activity was insensitive to amytal and rotenone, and was inhibited by excess NADH and *p*-chloromercuriphenylsulfonate (PCMS) as shown in Table II. It is of interest to note that the enzyme was more sensitive to higher levels of NADH in comparison to Singer's enzyme[8]. NADH, when present at a concentration as low as 0.11 mM, was inhibiting while Singer's enzyme showed maximum activity at an NADH concentration near 0.15 mM[8]. On the other hand, the sensitivity of NADH dehydrogenase to sulfhydryl inhibitors, which depends

TABLE II

INACTIVATION OF NADH DEHYDROGENASE BY NADH AND PCMS

NADH		*PCMS***	
Concn. (mM)	*Activity**	*Concn. (μM)*	*Inhibition (%)*
0.038	0.137	33	15
0.075	0.148	132	32
0.113	0.113	330	60
0.150	0.097		
0.225	0.072		
0.900	0.024		

*Activity was expressed as the decrease of absorbance at 420 nm per min, using 20 μg of NADH dehydrogenase and 0.833 mM ferricyanide as acceptor.

**An aliquot of NADH dehydrogenase (6.0 μg) was incubated with PCMS in 40 mM triethanolamine–HCl buffer, pH 7.8, at 30°, for 5 min before assay as described by Minakami *et al.*[12]. The activity without inhibitor was 100 μmoles NADH oxidized per min per mg.

to a great extent upon the buffer and temperature of preincubation[12] was less. Thus approximately 30% inactivation of our enzyme was effected by 132 μM PCMS, while Singer's enzyme was reported to require only 1 μM PCMS for the same degree of inactivation[12]. The low sensitivity towards PCMS was perhaps due to the presence of detergent in our preparation. Ferricyanide reduction activity was also destroyed upon treatment with ionic detergents, *e.g.* no appreciable activity of the enzyme could be measured after sodium dodecyl sulfate or sodium lauroyl sarcosinate (Sarkosyl NL-30, Geigy Industrial Chemicals) treatment (1%, 10 min, at 0°). However, non-ionic detergents seemed to have no effect on ferricyanide reduction activity; V_∞ was unchanged when 0.1% of Lubrol was present in the assay medium.

The presence of an iron–sulfur protein in the NADH dehydrogenase preparation was indicated by the high non-heme iron content and the appearance on reduction of a pronounced $g = 1.94$ EPR signal as shown in Fig. 2. The EPR spectrum was similar to that obtained from Singer's enzyme and Complex I (NADH–coenzyme Q reductase), which is a particulate preparation from beef heart mitochondria composed of a flavoprotein, a non-heme iron protein, some "structural-type" protein, ubiquinone and other lipids[13].

Some efforts have been directed towards the resolution of the NADH dehydrogenase with $NaClO_4$, one of the reagents used by Davis and Hatefi[14] for resolution of Complex I. That resolution indeed occurred during treatment of the NADH dehydrogenase with perchlorate was indicated by the loss of NADH–ferricyanide reductase activity which was paralleled by the appearance of NADH–menadione reductase activity. The low activity with ferricyanide and high activity with menadione are characteristic of most low-molecular-weight type NADH dehydrogenase preparations[1]. Resolution was effected most efficiently by the incubation of the enzyme with 1 M perchlorate at room temperature for a short time; 15 min incubation resulted in a 4-fold increase and a 65% decrease of menadione and ferricyanide reductase activities, respectively.

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