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Isolation and Characterization of a NADH-Dehydrogenase From Rat Liver Mitochondria

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Mitochondrial NADH dehydrogenase has been purified from rat liver mitochondria by protamine sulfate fractionation and DEAE-Sephadex chromatography. The enzyme is water-soluble and its molecular weight has been estimated at 400 ± 50 kilodaltons. NADH-ferricyanide reductase and NADH cytochrome c reductase activities have been studied and the kinetic parameters have been determined. Both substrates, NADH and the electron acceptor (ferricyanide or cytochrome c) have an inhibitor effect on the reductase activities and the kinetic mechanism of the enzyme is ping-pong bi-bi.

Key words: NADH-dehydrogenase, Liver mitochondria, Rat.

Since WARBURG and CHRISTIAN (21) revealed that flavoproteins were involved in the catalytic oxidation of NADH in mitochondrial respiration, the isolation of native NADH dehydrogenase has been attempted by different methods.

Among mammalian preparations, NADH dehydrogenase from beef heart mitochondria is the best studied; three preparations having reductase activity and differing in their solubility or molecular weight have been described.

Complex I (NADH-ubiquinone reductase) is a high molecular weight particle form first isolated by HATEFI *et al.* (4) from bovine heart mitochondria using chaotropic agents. Three enzymic activities are present in this Complex: NADH-reductase, NADH-reductase and NADPH-NAD-transhydrogenase, the first one being the most relevant activity. *In vivo* the electron acceptor is ubiquinone-10; *in vitro* ferricyanide may be used as acceptor, a higher specific activity being obtained; however, with other acceptors the specific activity is much lower (8, 9).

SINGER *et al.* (15) isolated a high molecular weight water-soluble form of NADH dehydrogenase, which they called NADH dehydrogenase-I, from beef heart mitochondria using *Naja naja* venom fosfolipase A. This preparation has

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a high NADH-ferricyanide reductase activity, but physiological NADH-ubiquinone reductase activity is absent (18).

A number of low molecular weight soluble preparations of the enzyme, NADH dehydrogenase-II, may be obtained from Complex I or from NADH dehydrogenase-I (6, 14). All these preparations are similar in composition and enzymic activity. They have a very low NADH-ferricyanide reductase activity (16); besides they have other activities not described for Complex I such as an antimycin resistant cytochrome c reductase activity, a high indophenol reductase activity and a ubiquinone reductase activity unaffected by rotenone (17, 22).

Soluble preparations of NADH-dehydrogenase from rat liver mitochondria have been obtained by different authors (13, 20); however, the liver enzyme is less known than that from other sources. In this paper a new method for the isolation and purification of a NADH-dehydrogenase from rat liver mitochondria is presented. Its catalytic properties, kinetic mechanism, as well as its possible relationship with the described forms of the mitochondrial enzyme are discussed.

Materials and Methods

Rat liver mitochondria were isolated by the method of HOGEBOOM (10).

The enzyme isolation was carried out as follows: The mitochondrial pellet from twelve rats was suspended in a final volume of 80 ml of a solution 2 mM ATP, 2 mM EDTA, 0.15 M sucrose, pH 7.8. The suspension was sonicated for 25 s in a beaker cooled in an ice bath, using a setting of 7 on a Branson sonifier equipped with a 0.5 inch step horn; it was then centrifuged at 78,000 g for 60 min at 0°C. The pellet was suspended in a volume of 50 ml of a solution 2 mM ATP, 2 mM EDTA, 0.15 mM sucrose, pH 7.8 at room temperature; pH was adjusted to 8.5 with Tris. Fractions of 25 ml were sonicated on a setting of 6 for 10 min and a setting of 7 for an additional time of 15 min. All the sonicated fractions were mixed and centrifuged at 85,000 g for 90 min at room temperature. The supernatant was collected and brought to pH 7.8 with acetic acid. The supernatant was fractionated by the slow addition of 40 μl per ml of salmon protamine sulfate (12.5 mg $\times$ ml^{-1}) (Sigma, Grade X). After stirring for 5 min, the suspension was centrifuged at 25,000 g for 10 min.

The NADH dehydrogenase contained in the supernatant solution was purified by ion-exchange chromatography. DEAE-Sephadex (A-25) was previously converted to its sulfate form and equilibrated with 50 mM Tris sulfate, pH 7.8. The column (2.6 $\times$ 11.3 cm) was prepared and 180 ml of 50 mM Tris sulfate, pH 7.8, passed through it, followed by 120 ml of 2 mM ATP, 1 mM EDTA, 50 mM Tris sulfate, pH 7.8. The enzymic preparation was added, and the column washed with 30 ml of the latter buffer. NADH dehydrogenase activity was eluted with 180 ml of the same buffer containing 30 mM K_2SO_4, at a flow rate of 60 ml/h. Fractions of 10 ml each were collected and the 2 or 3 tubes containing 75% or more of the eluted activity were pooled.

Protein determination was carried out following the technique of LOWRY *et al.* (12). SDS discontinuous gel electrophoresis was carried out following the technique of LAEMMLI (11) at 5-10 mA for 4-5 h. The gels were stained with Coomassie briliant blue as described by FAIRBANKS *et al.* (3). Cytochrome c, trypsin inhibitor, porcine and bovine serum albumin from Merck were used as standards in the molecular weight estimations.

NADH-ferricyanide reductase activity was determined spectrophotometrically in a cuvette containing 0.1 ml of enzymic preparation (40-100 μg of protein), 0.3 ml of 0.1 M Tris-acetic, pH 7.4, and 0.2 ml of each of the two substrates NADH and ferricyanide, at the appropriate concentrations; NADH oxidation was moni-

tored by the decrease of light absorption at 340 nm as described by HATEFI and RIESKE (5); the NADH extinction coefficient used was 6,220 M^{-1} cm^{-1}. NADH-cytochrome c reductase activity was measured by the increase of light absorption at 420 nm due to cytochrome c reduction. Reagent blanks were used in each experiment. The nonenzymic rate was determined and subtracted.

Results and Discussion

Isolation and characterization. — A new method of isolation of a NADH dehydrogenase activity from rat liver mitochondria is presented. This method is based on the breakage of mitochondrial membranes by sonication and selective solubilization of the enzyme by protamine sulfate fractionation, followed by ion-exchange chromatography.

The percent of recovery and the purification factor reached in each step is given in table I. Similar values have been obtained with 0.04 mM cytochrome c used as substrate.

A control of the enzymic nature of the NADH dehydrogenase activity was carried out; the incubation of the enzymic preparation at 50°C caused a loss of enzymic activity more acute when the incubation time was increased causing presumably the irreversible denaturation of the protein. With 0.528 mg protein × ml^{-1} and a final concentration of NADH and ferricyanide 0.08 mM, 20, 45 and 71 percent inhibition of the reductase activity is achieved after 10, 20 and 40 minutes respectively.

The SDS polyacrylamide gel electrophoresis of the purified enzyme revealed the existence of four polypeptides with molecular weights of about 14, 44, 72 and 97 kilodaltons respectively. Coomassie blue stain intensities of the bands suggest a ratio of 1:1 : 1:4. The minimum molecular weight of the enzyme calculated from these data was 400 ± 50 kilodaltons; this value falls within the range of the molecular weight previously assigned to NADH dehydrogenase-I from beef heart mitochondria (15).

Table I. *Summary of purification of rat liver NADH dehydrogenases NADH-ferricyanide reductase activity was determined as described and the final concentrations of NADH and ferricyanide were 0.4 mM.*

Step	% recovery	Purification factor
Solubilized NADH-dehydrogenase	—	—
Protamine sulfate fractionation	83.50	12.45
DEAE Sephadex chromatography	68.00	3.69

The high ferricyanide reductase specific activity of our preparation (334 μmoles of NADH oxidized per minute and milligram protein) also suggests a similarity to the NADH-dehydrogenase I isolated

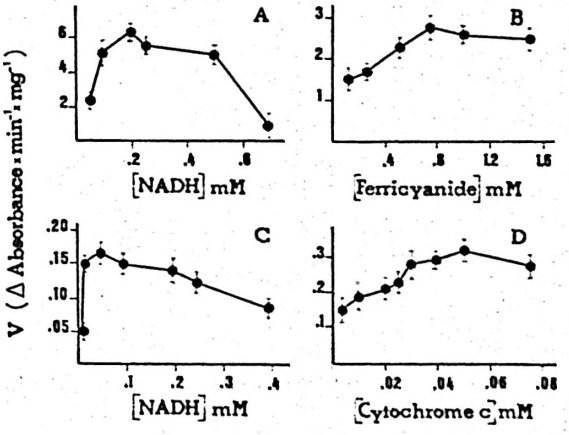

Fig. 1. *Effect of substrate concentration on the NADH-reductase activity.*
A) Effect of NADH concentration on the reduction of 0.25 mM ferricyanide. B) Effect of ferricyanide concentration on the oxidation of 0.20 mM NADH. C) Effect of NADH concentration on the reduction of 0.025 mM cytochrome c. D) Effect of cytochrome c concentration on the oxidation of 0.025 mM NADH. Number of experiments, 3.

by HATEFI from beef heart mitochondria (5).

NADH reductase kinetics. — Kinetic studies showed that there was a double inhibition of both NADH-ferricyanide and NADH-cytochrome c reductase activities (figure 1). NADH concentrations higher than 0.1 mM, and ferricyanide concentrations higher than 0.8 mM, inhibit NADH-ferricyanide reductase activity, similarly as reported by others (1, 7) for NADH dehydrogenase-I and Complex I respectively. The NADH cytochrome c reductase activity was inhibited by either NADH or cytochrome c concentrations higher than 0.05 mM. This double inhibition is a new support for the possible relationship between our preparation and NADH dehydrogenase-I isolated by other authors (1) from beef heart mitochondria.

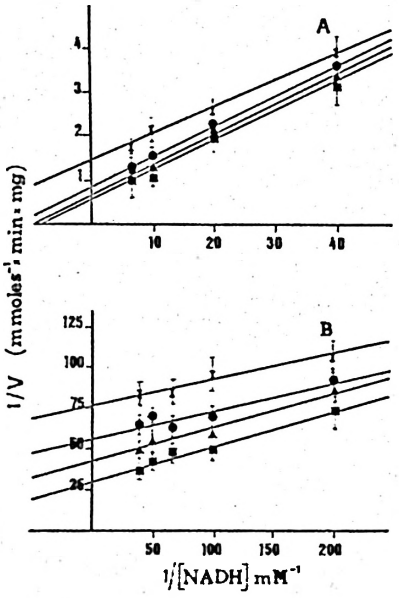

Fig. 2. *Kinetic mechanism of NADH-ferricyanide reductase and NADH-cytochrome c reductase activities.*

Lineweaver-Burk plot of initial rate of NADH-ferricyanide reductase and NADH-cytochrome c reductase activities as function of NADH concentration. A) At different concentrations of ferricyanide, (I) 0.1 mM (●) 0.25 mM, (Δ) 0.5 mM, (■) 0.75 mM. B) At fixed concentrations of cytochrome c, (I) 2.5 μM, (●) 10 μM, (Δ) 20 μM, (■) 25 μM. Number of experiments, 4. Correlation coefficients ≥ 0.9 have been obtained for the adjusted straight lines in all cases.

Table II. *Kinetic parameters of NADH-ferricyanide and NADH-cytochrome c reductase activities.* These values were calculated from figure 2.

	Km (μm)		Vmax (μmoles×min⁻¹×mg⁻¹)	
	NADH	Electron Acceptor	NADH	Electron Acceptor
NADH-ferricyanide reductase	90	60	595	334
NADH-cytochrome c reductase	4	8	34	42

The kinetic mechanism of the enzyme was ping-pong bi-bi as clearly shown by the pattern of the double reciprocal plot obtained at different substrate concentrations (figure 2). These results are in agreement with those previously reported by DOOIJEWAARD and SLATER (1) for NADH-dehydrogenase I from beef heart mitochondria.

Although NADH and the assayed acceptors have a very different chemical structure, the existence of a double inhibition by substrate, and the ping-pong mechanism, could be explained if it is assumed, as others have done (1, 2), that two different active centers could be present in the enzyme, but with a common access way to them.

The kinetic parameters obtained for NADH-ferricyanide and NADH-cytochrome c reductase activities are shown in table II. They are within the range previously described for the soluble high molecular weight forms of NADH dehydrogenase isolated from beef heart mitochondria (1, 7).

The results here reported indicate that the enzymic preparation obtained could

be related to the soluble high molecular weight NADH dehydrogenase from beef heart mitochondria (1, 7, 18). Similarities can be summarized as follows: both have similar molecular weights, a high NADH reductase specific activity, ping-pong bi-bi mechanism, double substrate inhibition and some similar kinetic parameters.

Resumen

Se describe un método de aislamiento y purificación de una NADH deshidrogenasa a partir de mitocondrias de hígado de rata basado en un fraccionamiento con protamina sulfato y una posterior cromatografía en DEAE-Sephadex. La enzima es soluble en agua y tiene un peso molecular aproximado de 400 ± 50 kilodaltons. Se han estudiado las actividades NADH-ferricianuro reductasa y NADH-citocromo c reductasa de la enzima y se han calculado sus parámetros cinéticos. Existe una doble inhibición por los sustratos NADH y aceptor electrónico (ferricianuro o citocromo c), y el mecanismo cinético de la enzima es ping-pong bi-bi.

Palabras clave: NADH deshidrogenasa, Mitocondrias.

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