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ELECTRON TRANSPORT SYSTEMS OF *CANDIDA UTILIS*

PURIFICATION AND PROPERTIES OF THE RESPIRATORY CHAIN-LINKED EXTERNAL NADH DEHYDROGENASE +

BRUCE MACKLER, BYRON HAYNES, RICHARD PERSON and GRAHAM PALMER

Department of Pediatrics and Center for Child Development and Mental Retardation, University of Washington, Seattle, WA, and Department of Biochemistry, Rice University, Houston, TX (U.S.A.)

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Summary

The respiratory chain-linked external NADH dehydrogenase has been isolated from *Candida utilis* in highly purified form. The enzyme is soluble and has a molecular weight of approx. $1.5 \cdot 10^6$. The enzyme contains two moles of FMN per mole of enzyme and is composed of two large subunits of mol. wt. 270 000 and eight smaller subunits of mol. wt. 135 000. Iron and copper are present in the preparations, but appear to be contaminants. The enzyme catalyzes the oxidation of NADH and NADPH at nearly equal rates and reacts readily with 2,6-dichlorophenolindophenol, CoQ_6 and CoQ_1 derivatives as acceptors. Rotenone (10^{-5} M) and seconal (10^{-3} M) do not inhibit enzymatic activity.

Introduction

Previously we have described the preparation and some properties of an electron transport particle from *Candida utilis* [1]. This preparation more closely resembled similar preparations from beef heart muscle than from *Saccharomyces cereviciae* since (i) the *C. utilis* enzyme contained both FMN and FAD, (ii) the NADH oxidase activity was inhibited by rotenone and seconal, and (iii) the preparations contained a nonheme iron component which appeared to be associated with the NADH dehydrogenase portion of the system. Ohnishi et al. [2] and more recently Von Jagow and Klingenberg [3] have presented data which

demonstrates that there are two NADH dehydrogenases associated with the inner mitochondrial membranes of *C. utilis* and *Saccharomyces carlsbergensis*, one of which is externally oriented (rotenone insensitive) and the other internally oriented (rotenone sensitive in *C. utilis* only).

The present communication describes the purification and properties of the external, respiratory chain-linked NADH dehydrogenase of *C. utilis* and presents evidence that the dehydrogenase is solubilized during preparation of electron transport particles from *C. utilis*.

Methods and Materials

Cultures of *C. utilis* were obtained from the American Type Culture Collection (ATCC No. 8205) and were grown in a medium containing 1% dextrose, 1% yeast extract (Difco) and 2% peptone (Difco) in a 130 liter New Brunswick Fermacell Fermentor at 30°C with rapid stirring (200 rev./min) and maximum aeration. Dow antifoam A was used to prevent foaming. The yeast were harvested in late stationary phase of growth (over 7 h in stationary phase).

Mitochondria were prepared from the cultures of *C. utilis* by modifications of the methods of Ohnishi [4] and Kovac et al. [5] using glusulase digestion as described below. Ten grams of yeast (wet weight) were suspended in 35 ml of a solution of 1.3 M sorbitol, 0.1 mM disodium (ethylenediamine)tetraacetate (EDTA), and 10 mM Tris-HCl of pH 5.7 and centrifuged for 5 min at $5100 \times g$. The yeast were resuspended in a solution of 0.15 M mercaptoethanol and 10 mM Tris of pH 8.8 (20 ml per g of yeast) and incubated at 30°C for 20 min. The suspension was then centrifuged for 5 min at $5100 \times g$ and the yeast were resuspended in 120 ml of the solution of 1.3 M sorbitol, 0.1 mM EDTA and 10 mM Tris-HCl of pH 5.7. The yeast were washed two times more by centrifugation and resuspension in the sorbitol, EDTA, Tris solution and were finally resuspended in the same solution (2 ml per g of yeast). Glusulase (14 mg of protein per g of yeast) was added to the yeast suspension which was then incubated at 30°C for approx. 90 min (the optimum time of incubation varied for each batch of yeast). The suspension was diluted to a volume of 50 ml with a solution of 1.3 M sorbitol, 0.1 mM EDTA, 10 mM Tris-HCl and 5 mM $MgCl_2$ of pH 5.7 and the suspension was centrifuged for 5 min at $4000 \times g$. The pellet was gently resuspended in 60 ml of the solution of sorbitol, EDTA, Tris and $MgCl_2$ and was again centrifuged for 5 min at $4000 \times g$. The pellet was then suspended at 0—5°C in a solution (10 ml per g of yeast) of 0.4 M mannitol, 0.5 mM EDTA, 10 mM Tris, and 5 mM $MgCl_2$ of pH 6.8 which contained 0.5 mg per ml of bovine serum albumin (fraction V). All subsequent procedures were performed at 0—5°C. The suspension was homogenized for 5 s at medium speed in a Virtis homogenizer, and was then centrifuged 10 min at $3000 \times g$. The pellet was discarded and the supernatant suspension was centrifuged for 10 min at $5500 \times g$. The resulting pellet was suspended in 30 ml of a solution (pH 6.8) containing 0.6 M mannitol, 0.5 mM EDTA, 10 mM Tris, 5 mM $MgCl_2$, and 0.5 mg/ml of albumin, was recentrifuged for 10 min at $5500 \times g$, and the final pellet of mitochondria was gently suspended in a minimal amount of the same solution (10 to 15 mg of protein per ml).

Studies of oxidative phosphorylation were performed at 25°C by means of a

Clark oxygen electrode and measurement of ^{33}P incorporation as described previously [6], except that the assay mixture contained 1.2 ml of a solution (pH 6.8) of 0.75 M mannitol, 1.5 mM EDTA, 50 mM Tris, 10 mM KCl and 10 mM $MgCl_2$; 0.2 ml of a 5% solution of bovine serum albumin (purified); 0.1 ml of a 1% solution of hexokinase; 0.10 ml of a solution of 0.15 M ^{33}P (pH 6.8), 20 mM ADP, 0.2 M glucose and 10 mM NADH or NADPH; and approx. 0.5 mg of mitochondrial protein.

Assays for NADH and NADPH oxidase activities and reductase activities with 2,6-dichlorophenolindophenol, CoQ_{10}, CoQ_6, CoQ_1 analog (2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone), cytochrome *c* and ferricyanide as acceptors were performed at 25°C as described previously [7—9].

Flavin was released from the enzyme preparations and identified by ascending paper chromatography and fluorescence intensity as described previously [8,10,11]. FMN concentrations were determined by the decrease in absorbance at 462 nm of preparations of the enzyme or at 450 nm of extracts of the flavin after addition of hydrosulfite using a millimolar difference extinction coefficient of 11.3 [8,10].

Iron, copper and zinc were determined with a Perkin-Elmer atomic absorption spectrophotometer equipped with a graphite furnace. The enzyme preparations were dialyzed for 48 h against 4 changes of 1 l each of a solution (pH 7.5) containing 0.005 M EDTA and 0.01 M Tris acetate before metal analyses were performed.

EPR Spectra were recorded on the undialysed enzyme at 12 K using a Varian E-6 X-Band spectrometer. The modulation amplitude used was 12 gauss and microwave power was varied from 8 μwatts to 2 mwatts as needed. Routine scans were performed from 200—4200 gauss; the $g = 2$ region at 3280 gauss was also examined at an expanded sweep.

The molecular weight and purity of the enzyme preparations were determined by polyacrylamide gel electrophoresis as described previously by Hedrick and Smith [12] with molecular weight standards obtained from Pharmacia Fine Chemicals (Piscataway, NJ). The subunit structure of the preparations was determined by electrophoresis in sodium dodecyl sulfate as described previously by Weber and Osborn [13]. Gels were fixed, stained with Coomassie Brilliant Blue R, and destained in an Isco destainer. The gels were scanned at 580 nm in an Isco linear gel scanner equipped with an integrating recorder.

Protein was determined by the method of Lowry et al. [14]. NADH, NADPH, bovine serum albumin, cytochrome *c*, 2,6-dichlorophenolindophenol, ADP, rotenone, CoQ_{10}, CoQ_6, and SDS were obtained from Sigma Chemical Company. Antimycin A was a gift from Ayerst Labs. The CoQ_1 analog was kindly given us by Dr. T.P. Singer. Glusulase was obtained from Endo Laboratories, and ^{33}P from New England Nuclear Company.

Results and Discussion

Purification of the external NADH dehydrogenase

Electron transport particles were prepared from *C. utilis* as described previously by Mahler et al. [15]. During the course of preparation of the electron transport particles, assays of NADH oxidase activity demonstrated that the

intact mitochondrial fraction isolated as fraction R_1 in the procedure was only inhibited 60% by rotenone ($5 \cdot 10^{-5}$ M), whereas the following fractions containing the electron transport particles and broken mitochondria (R_2L and R_2H) were inhibited 95—100%. (Assays were performed with and without added deoxycholate with the same results to reduce the effects of 'sidedness' of the preparations.) This suggested strongly that the rotenone insensitive external NADH dehydrogenase was detached from the inner mitochondrial membranes during breakage of the mitochondria and was present in the supernatant fraction S_2. Assays of the supernatant fraction S_2 confirmed the presence of a soluble NADH dehydrogenase which reacted readily with CoQ_6 and CoQ_1 analog and with indophenol as acceptors. Further purification of the enzyme was performed at 0—5°C as follows. Streptomycin sulfate was added to approx. 1400 ml of supernatant fraction S_2 to a final concentration of 0.8 g/100 ml, the suspension was stirred for 10 min and then centrifuged in the 30 rotor of a Spinco ultracentrifuge for 60 min at 30 000 rev./min. The residue was discarded and the supernatant solution (S_3) was made 80% saturated by addition of solid ammonium sulfate (51.6 g/100 ml). The suspension was stirred for 20 min and then centrifuged in the GS-3 rotor of a Sorvall refrigerated centrifuge for 20 min at 8800 rev./min. The residue (R_1) was resuspended in 0.02 M potassium phosphate buffer pH 8.0 (approx. 250 ml) and a saturated solution of ammonium sulfate (pH 8.0) was added slowly with stirring to a final concentration of 40%. After stirring for 5—10 min, the suspension was centrifuged in the GSA rotor of the Sorvall centrifuge for 20 min at 10 000 rev./min, the residue was discarded and the supernatant solution was made 65% saturated by a further addition of saturated ammonium sulfate. After stirring for 5 min, the suspension was again centrifuged for 20 min at 10 000 rev./min in the Sorvall centrifuge and the residue (R_2) was dissolved in 0.02 M potassium phosphate buffer of pH 8.0 (approx. 80 ml). The resulting clear solution was similarly fractionated by addition of the saturated solution of ammonium sulfate (pH 8.0) to 43% saturation and then to 49% saturation. The residue (R_3) obtained after centrifugation of the 49% saturated suspension contained the active NADH dehydrogenase and was dissolved in 0.02 M potassium phosphate buffer pH 8.0 (approx. 3.0 ml) and stored at −20°C until the next procedure. After thawing, the enzyme preparation was carefully layered onto a 27 ml sucrose

TABLE I

PURIFICATION OF THE EXTERNAL NADH DEHYDROGENASE

+ Specific activity, μmol of NADH oxidized per min per mg of protein.

Fraction	Total protein (mg)	Specific activity (indophenol)	Total activity (indophenol)	% recovery of activity
S_3	10 325	0.087	898.3	100
R_1	1 896	0.17	322.3	36
R_2	992	0.32	317.4	35
R_3	187	1.72	321.6	36
After gradient final prep.	14	8.50	119.0	13

gradient solution (pH 8.0) contained in a 30 ml centrifuge tube for the SW 25.1 rotor of the Spinco ultracentrifuge. The gradient ranged from 0.5 M sucrose at the top to 1.2 M sucrose at the bottom and contained 0.01 M potassium phosphate. The gradient tube was centrifuged for 17 h at 25 000 rev./min in the SW 25.1 rotor in a Model L2-65B Spinco ultracentrifuge. After centrifugation, the bottom of the gradient tube was pierced with an 18 gage needle and fractions of 0.6 ml were collected and assayed for NADH dehydrogenase activity. The most active fractions were combined and constituted the final preparation. Table I shows the activities and yields of the enzyme at various stages in the purification process.

Properties of the external NADH dehydrogenase

Preparations of enzyme of the highest activity catalyzed the oxidation of 8.5, 0.9, and 0.8 μmol of NADH per min per mg of enzyme protein when indophenol, CoQ_1(DPB) and ferricyanide, respectively, were used as acceptors. The enzyme also slowly catalyzed the oxidation of NADH when CoQ_{10}, CoQ_6, cytochrome *c* and O_2 were used as acceptors, but at rates less than 5% that with indophenol. In addition, the enzyme catalyzed the oxidation of NADPH with all acceptors at approximately 75% the rate of oxidation of NADH. When cytochrome *c* was used as acceptor and NADH as substrate, addition of superoxide dismutase ($2 \cdot 10^{-6}$ μmol) to the assay had no effect on enzymatic activity implying that the reduction of cytochrome *c* by the enzyme was not mediated by the superoxide radical. Enzymatic reactions with indophenol had a pH maximum between 6 and 7 and between 6.5 and 8.5 when CoQ_1 analog was used as acceptor. The enzyme did not catalyze the reduction of added FAD or FMN and activity was not stimulated by their addition. The Michaelis constant (K_m) of the enzyme for NADH determined with indophenol as acceptor by the method of Lineweaver and Burk [16] was calculated to be $4.3 \cdot 10^{-5}$ M.

Since the purified dehydrogenase oxidized NADH and NADPH at nearly the same rates, it was of importance to determine if the external pathways of NADH and NADPH oxidation and phosphorylation were similar in intact mitochondria. The results of such studies are shown in Table II and demonstrate that intact mitochondria from *C. utilis* oxidize added NADH and NADPH at relative rates similar to those for the purified dehydrogenase and with P : O ratios for both NADH and NADPH of approx. 2. The results confirmed that the purified enzyme was indeed the external respiratory chain-linked NADH dehydrogenase since the preparations retained the properties of the intact mitochondrial system.

TABLE II

STUDIES OF OXIDATIVE PHOSPHORYLATION WITH *C. UTILIS* MITOCHONDRIA

Substrate	Additions	Oxygen utilized *	P:O ratio
NADH	—	0.233	1.8
NADH	10^{-5} M rotenone	0.156	1.7
NADPH	—	0.153	1.7
NADPH	10^{-5} M rotenone	0.131	1.6

* μatoms per min per mg of mitochondrial protein.

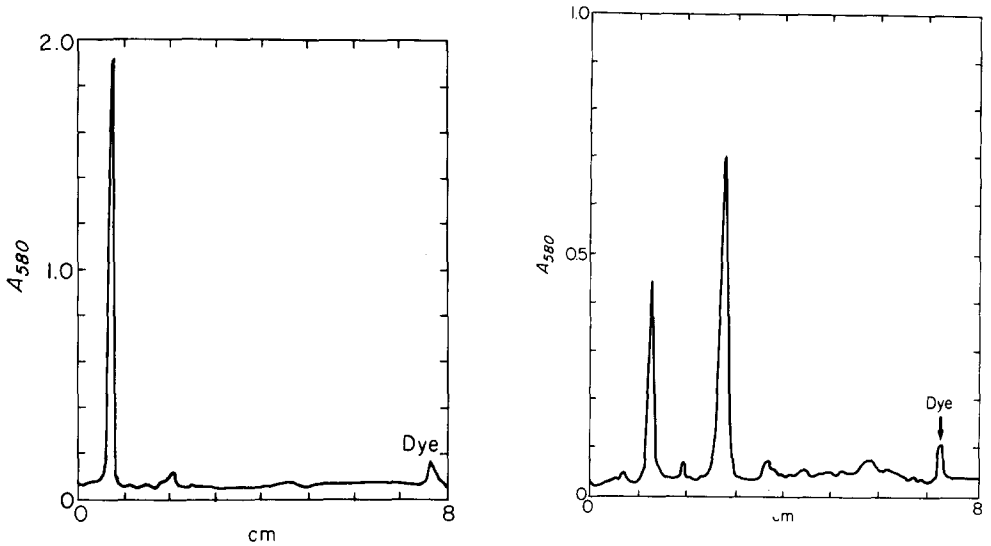

Fig. 1. Densitometric trace of a preparation of the external NADH dehydrogenase electrophoresed on a 3% polyacrylamide gel. 0.01 mg of enzyme protein was used in the study.

Fig. 2. Densitometric trace of the external NADH dehydrogenase electrophoresed in SDS on a 3.3% polyacrylamide gel. 0.02 mg of enzyme protein was used in the study.

When preparations of the highest activity were studied by acrylamide gel electrophoresis the protein moved as a single symmetrical peak as shown in Fig. 1, demonstrating that the preparations were over 90% homogeneous. Because of the high molecular weight, migration was small even in the 3.0% gel but in all cases the preparation clearly moved into the gels. The enzyme was calculated to have a molecular weight of (1.5—1.6) · 10^6 in other electrophoretic studies performed by the method of Hedrick and Smith [9] using gels which ranged from 3—4%. When preparations of the enzyme were studied by electrophoresis in sodium dodecyl sulfate as shown in Fig. 2, it was found that two peaks were now present representing two species of subunits with molecular weights of 270 000 and 135 000, respectively. Integration of the area under the peaks by means of an integrating Houston recorder showed that the peak representing the smaller subunit contained two times the amount of protein (assuming equal dye binding) than the peak of the larger subunit and indicated that the subunits were present in the enzyme in a ratio of one large subunit : four small subunits for a total of two large subunits and eight small subunits per mol of enzyme.

Preparations of enzyme of the highest purity contained flavin, copper and iron in an approximate ratio per mol of enzyme (mol. wt. 1.5 · 10^6) of 2 : 2 : 1.5, respectively, as shown in Table III. Varying amounts of zinc were found in the enzyme preparations (0.1—1.0 mol per mol of enzyme) but since concentrations of zinc were very low in most of the preparations it was regarded as a contaminant. Since the preparations contain 2 mol of flavin per mol of enzyme (see Table III), it would appear that one mol of flavin may be associated with

TABLE III

COMPOSITION OF THE EXTERNAL NADH DEHYDROGENASE

Preparation	Flavin * (calculated as FMN)	Copper *	Iron *	Specific activity ** CoQ_1 (DPB)
1	1.97	3.05	1.18	0.4
2	1.85	1.58	2.69	0.6
3	2.09	1.59	0.91	0.4
4	1.82	0.48	0.73	0.5
5	1.98	3.08	2.69	0.5
6	1.89	3.06	1.20	0.9
7	1.88	2.51	0.38	0.6
8	1.85	2.36	0.57	0.9
Average	1.92 ± 0.03	2.21 ± 0.33	1.29 ± 0.32	0.6 ± 0.08

* All results are expressed as mol per mol of enzyme ± the standard error of the mean, assuming a mol. wt. of $1.5 \cdot 10^6$ for the enzyme.

** Specific activity, μmol of NADH oxidized per min per mg of enzyme protein.

each of the 2 large subunits of the enzyme. The EPR spectrum of the resting undialyzed enzyme showed only one absorbance feature in the range $g = 1.6$–32; this feature was centered at $g = 2.1$ and had a lineshape consistent with its assignment as Cu (II). The resonance saturated very easily and the signal was too weak to run under non-saturated conditions at this, or at higher, temperatures. However, an estimate of the concentration of Cu (II) using Cu (II)-EDTA as an intensity standard led to a value of 7 μM; the concentration of cupric ion determined chemically was 15 μM. This result suggests that a large fraction of the chemically determined cupric ion is contributing to the EPR spectrum. The EPR spectrum did not exhibit any features at $g = 6$ nor at $g = 4.3$; the instrument noise was less than 2% of the total amplitude of the signal amplitude at $g = 2$. Addition of an excess of NADH to the enzyme sample did not lead to

TABLE IV

CHARACTERISTICS OF THE FLAVIN COMPONENT OF THE EXTERNAL NADH DEHYDROGENASE

Compound	Chromatography solvent systems		Relative fluorescence intensity *
	n-butanol/acetic acid/water $R_F \times 100$	5% Na_2HPO_4 $R_F \times 100$	
FAD	5	39	0.1
FMN	13	56	1.0
Dehydrogenase flavin	11	55	0.7
FAD + dehydrogenase flavin	Two spots	Two spots	
	5	40	—
	10	56	
FMN + dehydrogenase flavin	One spot	One spot	
	11	54	—

* Fluorescent studies were performed with a 1.0 mM solution of each of the compounds in a Farrand Spectrofluorometer.

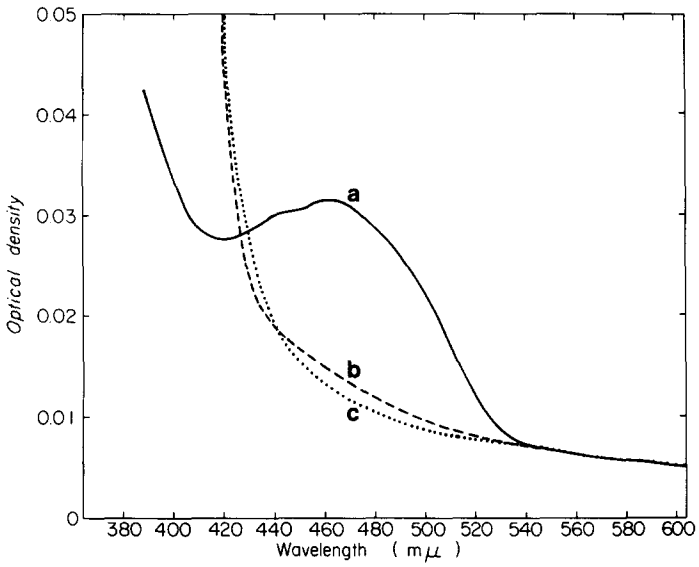

Fig. 3. Spectra of a preparation of external NADH dehydrogenase in (a) oxidized state, (b) after reduction by addition of approx. 1 mg of solid NADH, and (c) after reduction by addition of 1—2 mg of hydrosulfite. The sample cuvette contained 1.35 mg of enzyme protein per ml of 0.02 M potassium phosphate buffer of pH 7.5. The blank cuvette contained only the phosphate buffer.

the production of any new signals, and only a 20% decrease in the amplitude of the copper resonance was observed. No additional decrease occurred even after a 10-min incubation at room temperature. The same result was obtained whether or not the enzyme had been frozen prior to addition of the NADH. Addition of solid dithionite to the enzyme led to the complete disappearance of the copper signal; no signal of the $g = 1.94$ type was produced with this reagent.

The EPR data, together with the observed variability in the chemical analyses for iron and copper and the lack of correlation of the metal content with enzymatic activity (see Table III), suggest that the two metal ions are probably not active components of the enzyme. The small decrease in the intensity of the copper EPR signal after addition of NADH was probably artifactual. The turnover number of the enzyme with NADH is at least 100 s^{-1} as measured by the indophenol assay and thus all redox active components should be reduced extremely rapidly.

The flavin was completely released from the enzyme by first heating the preparation at 100°C for 6 min and then by addition of perchloric acid to a final concentration of 10% [8]. Analysis of the flavin by chromatographic techniques and by comparison of its fluorescence intensity with standards of FMN and FAD demonstrated that the flavin component was FMN as shown in Table IV. Fig. 3 shows the spectra of a preparation of purified enzyme in the oxidized state and after reduction by NADH or hydrosulfite. As shown in this figure, NADH addition reduced the FMN component over 90% compared with hydrosulfite as determined by the decrease in absorbance at 462 nm and dis-

appearance of the absorption peak which represents the FMN component.

Finally, the effects of inhibitors were studied on the enzyme preparations. Bathocuproine ($5 \cdot 10^{-5}$ M) and antimycin A (0.03 mg) when added to the assay system inhibited enzymatic activity over 80% (without preincubation) with CoQ_1 analog as acceptor but had no effect when indophenol was used as acceptor. Dithizone ($5 \cdot 10^{-5}$ M), arsenite (10^{-3} M), *o*-phenanthroline ($5 \cdot 10^{-5}$ M), *p*-chloromercuriphenyl-sulfonic acid (10^{-4} M), rotenone (10^{-5} M), seconal (10^{-3} M), and dicumarol (10^{-6} M) had no effect on enzymatic activity with either acceptor.

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