VARIATION IN INHIBITOR SENSITIVITY OF NADH-MENADIONE REDUCTASE FROM MITOCHONDRIA

C. L. Hall and F. L. Crane
Department of Biological Sciences
Purdue University
Lafayette, Indiana

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The presence of an amytal-sensitive NADH-menadione reductase in beef heart mitochondria has been previously reported (Cunningham, et al., 1965; Cunningham, et al., 1965b; Crane, et al., 1965). The enzyme described was found in the aqueous supernatant after extraction of beef heart mitochondria with diethyl ether. The enzyme has been further studied and purified and has been found to undergo changes during purification procedures, as shown by its response to different inhibitors. We present data which describe some of the characteristics of the enzyme and which suggest the enzyme is related to the electron transport chain activity in mitochondria.

METHODS AND MATERIALS

Beef heart mitochondria were prepared according to the method of Löw and Vallin (1963). Electron transport particles (ETP_H) were obtained by sonic disruption of mitochondria at 6-7 amperes (#8 setting, Branson sonifier).

Ether extraction was carried out at room temperature. 25-100 ml of ETP_H (25-40 mg protein/ml) were extracted 3 min with 5-10 vol ethyl ether.

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The particles were allowed to settle 1-2 min and the aqueous phase drawn off into 25-100 ml 0.25 M sucrose. The suspension was aspirated 15-20 min and centrifuged 30 min at 40,000 rpm in the Spinco Model L ultracentrifuge.

Polarographic assay of NADH oxidase activity and spectrophotometric assay of NADH-cytochrome c reductase and NADH-menadione reductase activities were carried out essentially as described by Cunningham, et al., (1965). NADH-ferricyanide reductase assay was carried out as described by Minakami, et al., (1962). Vitamin K_1 and K_2 analogues were a gift from Dr. O. Isler of Hoffmann-LaRoche and Co., Basle. Coenzymes Q_0 and Q_2, and piericidin A were a gift from Dr. K. Folkers, Stanford Research Institute, Menlo Park, California. All other chemicals were obtained commercially.

DEAE-cellulose was treated twice with 1 N HCl and the supernatant, including fine particles, was decanted. The suspension was washed twice with deionized water, decanting each time, and neutralized with 0.3 M Tris, followed by two washes with deionized water decanting as above each time. Finally, the suspension was washed and decanted 2 or 3 times with 0.003 M Tris-Cl pH 7.4. All preparations were carried out in the cold (0-4°).

Protein was determined by the biuret method (Gornall, et al., 1949) and by the Folin-Ciocalteu method of Lowry, et al., (1951).

RESULTS

Attempts were made to release the NADH-menadione reductase from ETP_H by means of digestion with phospholipase A, which was shown to release the NADH-ferricyanide reductase (Ringler, et al., 1960; Minakami, et al., 1962). Some activity was always released by the digestion but attempts to separate the two activities resulted in a loss of amytal sensitivity of the NADH-menadione reductase.

It was found that the NADH-menadione reductase can be inhibited by piericidin, a new inhibitor of electron transport in beef heart mitochondria

(Hall, et al., 1966) and also by rotenone. Sensitivity to these inhibitors appeared to be modified less or not at all by treatments in which sensitivity to amytal was lost; and thus was used to follow the enzyme during purification. Chromatography of the enzyme on DEAE-cellulose resulted in a large or complete loss of the enzyme as judged by amytal sensitivity, but rotenone and piericidin sensitivities were decreased very little, and rotenone sensitivity often increased on purification. Using rotenone or piericidin sensitivities as criteria for the NADH-menadione reductase it was seen that the NADH-menadione reductase activity could be eluted from a DEAE-cellulose column at 0.15 M Tris-Cl pH 7.4 and that the fraction containing NADH-menadione reductase activity was now free of NADH-ferricyanide reductase activity. The NADH-menadione reductase is eluted as a single peak and is yellow in color. The peak in NADH-menadione reductase activity from the column parallels a peak in protein eluted, but protein continues to be eluted at lower levels in subsequent fractions. A dark yellow band remains on the column.

TABLE I

Purification of NADH-menadione Reductase

	NADH oxidation rate with menadione as acceptor				
Fraction	SA[1]	Total units per fraction	% activity released	Total protein (mg)	Total protein recovered
ETP	0.09	184	--	1980	--
Supt[2]	0.14	38	14.6	249	12.5
Purified Enzyme	1.48	17	9.4	11.7	4.7

[1]SA = specific activity μmoles/min/mg protein

[2]supt = aqueous supernatant after ether extraction

The specific activities of the different fractions and the percent recoveries of protein and activity are shown in Table I.

The visible spectrum of the enzyme showed some bleaching at 450 mμ on addition of NADH. Accordingly, the enzyme was assayed for flavin. Nearly all the flavin is acid-extractable (3.75 mμmoles/mg protein) as was shown for NADH-dehydrogenase from ETP by Blair et al., (1963). The enzyme was also assayed for total iron, presumed to be non-heme iron since the spectrum

TABLE II

Activity of NADH-menadione Reductase with Different Electron Acceptors

Electron acceptor	Concentration (M)	Activity (μmoles NADH oxidized/ min/mg protein)
Menadione	1.4×10^{-4}	0.36
Vitamin K_1	1.24×10^{-4}	1.41
Vitamin $K_{2(10)}$	1.02×10^{-4}	0.87
Coenzyme Q_0	1.4×10^{-4}	0.73[1]
Coenzyme Q_2	1.6×10^{-4}	0
	1.3×10^{-3}	0.11
Coenzyme Q_{10}	1.5×10^{-4}	0.03
	7.2×10^{-4}	0.22
DMBQ[2]	1.9×10^{-4}	1.15
TMBQ[3]	1.9×10^{-4}	0.64[1]
DQ[4]	1.5×10^{-4}	0.02
α-tocopherylquinone	1.29×10^{-4}	1.01

[1]Activity linear only for 30 sec. Rate dropped off rapidly after this time.

[2]DMBQ = 2,3,-dimethylbenzoquinone

[3]TMBQ = 2,3,5-trimethylbenzoquinone

[4]DQ = duroquinone = 2,3,5,6-tetramethylbenzoquinone

showed no indications of the presence of heme, and was found to contain 8.64 mμmoles/mg protein. The ratio of iron to flavin is thus 2.3.

The best conditions for activity and inhibition by rotenone were at a final molar concentration of 5×10^{-4} M Tris-Cl pH 7.4. The enzyme has no activity with NADPH used at the same concentration as NADH. The NADH menadione reductase was tested with different electron acceptors (Table II). The response of the enzyme with coenzyme Q_0 and with TMBQ as acceptors was different from the response with menadione in that activity was stimulated during the first 30 seconds of assay, but stopped altogether soon after that time. With dimethylbenzoquinone the activity was increased and remained linear during the time of assay of 1-2 minutes. Activity with coenzymes Q_2 and Q_{10} as acceptors was very low when assayed at approximately the same concentration as menadione. Some activity could be seen at higher

TABLE III

Inhibition of NADH-menadione Reductase by Different Agents

Inhibitor	Concentration	% Inhibition
Amytal	1.8×10^{-3} M	23
Dicoumarol	1.0×10^{-4} M	45
Mersalyl	1.8×10^{-6} M	33
Rotenone	5×10^{-9} moles/mg protein	0
	2.5×10^{-7} moles/mg protein	33
	2.5×10^{-6} moles/mg protein	83
	5×10^{-6} moles/mg protein	60
	2.5×10^{-5} moles/mg protein	0
Piericidin	6.6×10^{-10} moles/mg protein	61
	6.6×10^{-9} moles/mg protein	100
	6.6×10^{-8} moles/mg protein	90
	6.6×10^{-7} moles/mg protein	90

concentrations, however. Duroquinone showed very low activity. α-tocopherylquinone, vitamin K_1 and vitamin $K_{2(20)}$ used as acceptors had high activity.

The NADH-menadione reductase shows the highest degree of inhibition by piericidin and rotenone (Table III). It is less sensitive to dicoumarol, mersalyl and amytal. Titration of the NADH-menadione reductase with rotenone showed that a level of 2.5 - 5 x 10^{-6} moles/mg protein were needed for complete inhibition, but with piericidin 6.6 x 10^{-9} moles/mg protein were sufficient for complete inhibition. These values correspond to molar inhibitor/flavin ratios of 6.7 x 10^4 for rotenone and 1.6 for piericidin.

Bovine serum albumin (5 mg/3 ml assay mixture) increased the activity of the NADH menadione reductase more than 3-fold, and the stimulated activity in the presence of BSA still appeared to have the same inhibitor characteristics as the activity without BSA. Tween 40 had no effect on the activity used at levels from 0.1 to 1.0 mg/3 ml reaction volume. Aging at 0-4° or in frozen state decreased the specific activity of the NADH-menadione reductase as much as ten times. Treatment of the aqueous supernatant after ether extraction with ethanol, low pH and heating according to the method of Sanadi for extraction of the ubiquinone reductase resulted in loss of NADH-menadione reductase activity and a large increase in antimycin-insensitive NADH-cytochrome c reductase activity. The enzyme is not released by treatment with saturated aqueous solution of ether in which no ether phase is present, nor is the NADH-ferricyanide reductase of Ringler, et al., (1962) converted to NADH-menadione reductase activity by ether. The NADH-ferricyanide reductase is also insensitive to rotenone (Hall, 1966).

The NADH-menadione reductase activity appears to be related to the NADH oxidase activity in many different treatments (Table IV). It is present in ETP_H in a reasonable amount compared to the NADH oxidase activity and is removed or destroyed by treatments which decrease or inactivate NADH oxidase activity.

TABLE IV

Relationship of Enzyme Activities in Different Fractions

Fraction	NADH oxidized with different acceptors (mμmoles/min/mg protein)				
	K_3[1]	cyt c + antimycin	FeCN (V_{max})	O_2	O_2 + rotenone[4] % inhibition
BHM	49	51	18,000	296	80 - 100
Sonic-treated BHM	76	25	18,700	2,400	-
ETP_H	110	5	9,450	686	50 - 100
Res after ether extr.	0	2	33,000	40	0
Supt[2]	88[3]	37	20,000	--	-
NADH-K_3 reductase	59[3]	4	420	--	-

[1] amytal-sensitive activity

[2] aqueous supernatant after ether extraction

[3] rotenone sensitive activity

[4] 1.5×10^{-5} moles/mg protein

DISCUSSION

The NADH-menadione reductase released from ETP_H by ether extraction appears to be different from other NADH dehydrogenases studied and has characteristics which suggest a role in the electron transport system.

The enzyme can be partially released by ether extraction and by phospholipase A digestion, but not by treatment with detergents, non-polar solvents, physical treatments, or treatment of the particles with ether at a saturation level. (Hall, 1966)

The enzyme has considerable similarity to the ubiquinone reductase of Pharo and Sanadi (1964), Sanadi, et al., (1965) particularly with respect

to non-heme iron/flavin ratio, and inhibition by rotenone; but it cannot survive the treatments used in isolation of the ubiquinone reductase and has much less activity with cytochrome c as acceptor. Activity with cytochrome c as acceptor increases markedly after the heat treatment at low pH in the presence of ethanol.

The NADH-menadione reductase may be the site of action of piericidin, and rotenone as well, though the rotenone binding site may have been altered in the isolated enzyme.

The NADH-menadione reductase activity shows a positive correlation with NADH oxidase activity in many different treatments. It is not produced by ether treatments, nor is the NADH-ferricyanide reductase of Singer and associates converted to NADH-menadione reductase activity by ether under the conditions used for extraction of the NADH-menadione reductase. Studies with other acceptors suggest that the amount of enzyme seen in ETP_H relative to NADH oxidase activity may be much higher when the enzyme is functioning with the proper acceptor. Neither the tocopheryl-quinone nor the napthoquinones would appear to be the natural acceptor, since neither type of quinone has been found in significant quantity in mitochondria.

The specific activity of the purified enzyme is increased 10-fold over the specific activity of the ETP_H, and the flavin content is increased 10-fold also. The non-heme iron to flavin ratio is lower than that of the NADH-ferricyanide reductase and the same as the NADH-ubiquinone reductase. In terms of protein recovery the purification is 200 fold, which correlates with the increase in the amount of piericidin needed to inhibit the enzyme in relation to the amount required to inhibit NADH oxidase activity in ETP_H.

It has been shown by Minakami, et al., (1964) that there is a non-heme iron containing protein which apparently is not included in the NADH-ferricyanide reductase and which is sensitive to amytal and involved in electron transport in sequence after the NADH-ferricyanide reductase. We would like

to suggest that the NADH-menadione reductase described here may be related both to the extra non-heme iron site of Minakami, et al., (1964) and to the sites of inhibition of amytal, rotenone, and piericidin in the mitochondrial electron transport system. The enzyme protein is probably the same as the ubiquinone reductase of Sanadi, et al., (1965) but the different method of preparation leads to different acceptor specificity as well as the remarkable variations in inhibitor sensitivity. Piericidin A appears to be the most consistent inhibitor throughout the purification.

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