Biochimica et Biophysica Acta, 977 (1989) 62–69
Elsevier

BBABIO 43077

Purification of NADH-ferricyanide dehydrogenase and NADH-quinone reductase from *Escherichia coli* membranes and their roles in the respiratory chain

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(Received 25 April 1989)

Key words: NADH : quinone reductase; Respiratory chain; NADH-dehydrogenase; Flavoprotein; (*E. coli*)

The respiratory chain-linked NADH-quinone reductase (NQR) and NADH-ferricyanide dehydrogenase (NFD) were extracted from membranes of *Escherichia coli* by *n*-dodecyl octaethyleneglycol monoether detergent and purified by DEAE-Sephacel, DEAE-5PW and Bio-Gel HTP column chromatography. The purified NQR contained FAD as a cofactor, catalyzed the reduction of ubiquinone-1 (Q_1) and reacted with NADH, but not with deamino-NADH (d-NADH), with an apparent K_m of 48 μM. On the other hand, the purified NFD contained FMN as a cofactor, reacted with both NADH and d-NADH, and catalyzed the reduction of ferricyanide but not Q_1. NFD showed a high affinity for both NADH and d-NADH with a K_m of 7–9 μM. NFD was inactivated, whereas NQR was rather activated, by preincubation with an electron donor in the absence of electron acceptor. These properties were compared with those of activities observed with inverted membrane vesicles with special reference to the generation of inside-positive membrane potential ($\Delta\psi$). It was found that d-NADH-reactive FMN-containing NFD is a dehydrogenase part of energy-generating NADH-quinone reductase complex. The FAD-containing NQR was very similar to that purified by Jaworowski et al. (Biochemistry (1981) 20, 2041–2047), and reduced Q_1 without generating $\Delta\psi$.

Introduction

At least two immunologically distinct NADH-dehydrogenases are present in cytoplasmic membranes of *Escherichia coli* [1]. Ohnishi et al. [2] and Matsushita et al. [3] extended analyses of these two NADH-ubiquinone oxidoreductase systems by using inverted membrane vesicles and demonstrated that one enzyme system that oxidizes both NADH and deamino-NADH (d-NADH) involves [4Fe-4S]- and [2Fe-2S]-type iron-sulfur clusters and is coupled to the generation of proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$). It was designated as NADH dh-I. The other enzyme system, designated as NADH dh-II, oxidized NADH exclusively without leading to the generation of $\Delta\tilde{\mu}_{H^+}$ [2,3]. Young et al. [4,5] have already cloned and sequenced a gene encoding NADH-dehydrogenase (ndh) from *E. coli* and the enzyme has been purified from membranes of genetically amplified strain [6]. The purified enzyme consisted of a single polypeptide of 47 kDa and contained FAD as a cofactor. This enzyme was assigned to correspond to NADH dh-II [2,3]. NADH dh-I, however, has not been purified due to its unstable nature.

We attempted to isolate enzymes participating in the NADH-quinone reductase segment of the respiratory chain of *E. coli*. To ensure the detection of enzyme(s) catalyzing a part of the NQR, ferricyanide was also employed as an electron acceptor and we could purify two distinct enzymes from the membranes of *E. coli*. One enzyme, catalyzing NQR activity, contained FAD, whereas the other, catalyzing NFD activity, contained FMN as a cofactor. From the comparison of several properties of the purified enzymes with those of activities observed with the inverted membrane vesicles, the FMN-containing NFD was found to be a part of energy-generating NADH-quinone reductase complex. On the other hand, the FAD-containing NQR was very similar to that purified by Jaworowski et al. [6]. This

Abbreviations: NQR, NADH-quinone reductase (EC 1.6.99.5); NFD, NADH-ferricyanide dehydrogenase (EC 1.6.99.3); Q_1, ubiquinone-1; d-NADH, deaminoNADH (reduced nicotinamide hypoxanthine dinucleotide); $\Delta\tilde{\mu}_{H^+}$, proton electrochemical gradient; $\Delta\psi$, membrane potential; $C_{12}E_8$, *n*-dodecyl octaethyleneglycol monoether; oxonol VI, bis(3-propyl-5-oxoisoxazole-4-yl)pentamethine-oxonol.

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paper deals with the purification and characterization of two enzymes isolated from *E. coli* membranes.

Materials and Methods

Chemicals

Liponox DCH, an alkyl polyoxyethylene ether detergent [7] was kindly supplied by Lion, Kanagawa, Japan. *n*-Dodecyl octaethyleneglycol monoether ($C_{12}E_8$) was obtained from Nikko Chemicals, Tokyo, Japan. Ubiquinone-1 (Q_1) was kindly supplied by Eizai, Tokyo, Japan. Reduced nicotinamide hypoxanthine dinucleotide (d-NADH) and deoxyribonuclease I (type IV) were obtained from Sigma. Bis(3-propyl-5-oxoisoxazole-4-yl)pentamethineoxonol (oxonol VI) was obtained from Molecular Probes, OR. Other reagents used were of analytical grade.

Enzyme assays

NFD was assayed at 30 °C in the reaction mixture containing 25 mM Tris-HCl (pH 7.5), 20 mM $MgSO_4$, 0.2 mM NADH (or d-NADH), 1.0 mM ferricyanide and enzyme in a total volume of 1.0 ml. The reaction was started by the addition of enzyme. The activity was measured from the decrease in absorbance at 420 nm by using the absorption coefficient of 1.0 $mM^{-1} \cdot cm^{-1}$.

NADH-Q_1 reductase was assayed at 30 °C in the reaction mixture containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 0.02% Liponox DCH, 0.1 mM NADH, 8 μM Q_1 and enzyme in a total volume of 2.0 ml. The reaction was started by the addition of enzyme. The activity was measured by following the formation of reduced Q_1 from the changes in absorption difference at the wavelength pair, 242–270.5 nm, by using the absorption coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$ as described previously [8]. The activity was also measured from the decrease in absorbance at 340 nm by using the absorption coefficient of 6.81 $mM^{-1} \cdot cm^{-1}$ [9]. Since the membrane vesicles and crude extracts contain ubiquinol oxidase activity, the NQR activity of these fractions was assayed in the presence of 10 mM KCN.

For the activity toward menadione or dichlorophenol-indophenol (DCIP), 0.1 mM or 40 μM, respectively, was used and the activity was measured at 340 or 600 nm by using the absorption coefficient of 6.22 or 20.6 $mM^{-1} \cdot cm^{-1}$.

One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH (d-NADH) or the reduction of 1 μmol ferricyanide or Q_1 per min.

Bacterial growth and preparation of membrane vesicles

E. coli K12, C600 was grown aerobically at 30 °C in a medium containing 1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 0.4% K_2HPO_4 (pH 7.4). Cells were harvested by centrifugation at the late exponential phase of growth and washed twice with 50 mM potassium phosphate (pH 7.5)/5 mM $MgSO_4$. Washed cells were suspended in 50 mM potassium phosphate (pH 7.5)/5 mM $MgSO_4$/1 mM dithiothreitol/10 μg deoxyribonuclease I per ml/10% (w/w) glycerol and the suspension was passed through a French pressure cell (Aminco) at 14000 psi. Unbroken cells and cell debris were removed by centrifugation at 30000 × *g* for 15 min, and the supernatant was centrifuged at 70000 × *g* for 2 h to sediment membrane vesicles. The membrane vesicles were washed once and suspended in 50 mM potassium phosphate (pH 7.5)/5 mM $MgSO_4$/10% glycerol and stored at −80 °C.

Extraction and purification of enzymes

About 1 g protein of the membrane vesicles was suspended in 250 ml of 20 mM Tris-HCl (pH 7.5)/5 mM EDTA/20% glycerol and it was mixed with a stirrer for 20 min at 4 °C. The mixture was centrifuged at 300000 × *g* for 1 h and the pellet was resuspended in 250 ml of 20 mM Tris-HCl (pH 7.5)/1 mM EDTA/20% glycerol (EDTA-washed membranes). To the suspension, $C_{12}E_8$ was added to give a final concentration of 2 mM and the mixture was incubated for 20 min at 4 °C. It was centrifuged at 300000 × *g* for 1 h and the supernatant was collected ($C_{12}E_8$ extracts).

The $C_{12}E_8$ extracts (400 mg protein) were applied to a DEAE-Sephacel column (3.2 × 10 cm) equilibrated with 10 mM Tris-HCl (pH 7.8)/0.5 mM $C_{12}E_8$/0.1 mM EDTA/10% glycerol (buffer 1) containing 50 mM NaCl. The column was washed with buffer 1 containing 100 mM NaCl until the absorbance of eluate at 280 nm decreased to a low level. Then the column was eluted with 250 mM NaCl in buffer 1. The active fractions were combined, then concentrated and desalted by ultrafiltration with an Amicon PM 10 membrane (DEAE-Sephacel fraction).

The DEAE-Sephacel fraction was applied to a TSK-gel DEAE-5PW column (2.15 × 15 cm) equilibrated with 10 mM Tris-HCl (pH 7.8)/0.1% Liponox DCH/0.1 mM EDTA/5% glycerol (buffer 2). The column was washed with 150 mM NaCl in buffer 2 and then eluted with a linear gradient from 150 to 400 mM NaCl in buffer 2 at a flow rate of 3 ml/min (see Fig. 1). The activity was separated into two fractions. The first fraction eluted at about 260 mM NaCl contained NFD activity and the second fraction eluted at about 320 mM NaCl contained NQR activity. The NFD and NQR fractions were separately combined, then concentrated, desalted and the concentration of Liponox DCH was decreased to 0.02% by ultrafiltration.

The NQR fraction was rechromatographed on DEAE-5PW under the same conditions as described above (second DEAE-5PW).

The NFD fraction was applied to a Bio-Gel HTP column (1.6 × 6.6 cm) equilibrated with 10 mM Tris-HCl

(pH 7.5)/10 mM NaCl/0.02% Liponox DCH/0.1 mM EDTA/5% glycerol/10 μM FMN (buffer 3). The column was washed with buffer 3 and then eluted with a linear gradient of sodium phosphate (pH 7.5) from 0 to 0.1 M in buffer 3 at a flow rate of 50 ml/h. The active fractions were combined, then concentrated and desalted by ultrafiltration (Bio-Gel HTP).

Measurement of membrane potential

The generation of membrane potential, positive inside, in the membrane vesicles was monitored by an absorbance band shift of oxonol VI [10,11] at 625–587 nm with a Hitachi 557 two-wavelength spectrophotometer. For this experiments, the inverted membrane vesicles were prepared by the French press at a shear force of 5000 psi. The reaction mixture contained 50 mM potassium phosphate (pH 7.5)/5 mM $MgSO_4$/10 mM KCN/2 μM oxonol VI/10 μM Q_1/0.1 mM NADH or d-NADH/200 μg protein of membrane vesicles in a total volume of 2.0 ml. The reaction was started by the addition of NADH or d-NADH at 20 °C. For the preincubation with an electron donor, NADH or d-NADH was added in the absence of Q_1 and then after indicated time intervals the reaction was started by the addition of Q_1.

Other methods

Flavins were extracted from the enzyme preparation by boiling for 5 min at neutral pH and then FMN and FAD contents were determined by the method of Faeder and Siegel [12]. Since the NFD fraction of Bio-Gel HTP contained free FMN, it was removed by the gel filtration on a TSK-gel G3000SWG gel column using a buffer system containing 10 mM Tris-HCl (pH 7.5)/40 mM NaCl/0.02% Liponox DCH/0.1 mM EDTA/5% glycerol at a flow rate of 3 ml/min.

SDS-polyacrylamide gel electrophoresis was performed by using discontinuous buffer system of Laemmli [13].

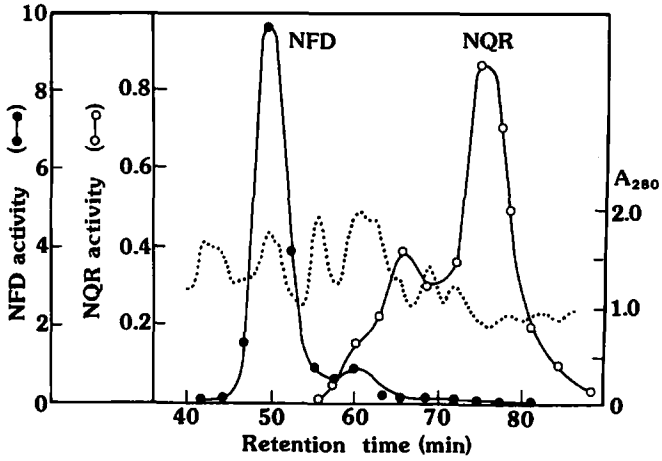

Fig. 1. First TSK-gel DEAE-5PW chromatography. About 75 mg protein of DEAE-Sephacel fraction was applied to the column and it was eluted by a linear gradient from 150 to 400 mM NaCl at the flow rate of 3 ml/min. The eluates were assayed for NADH-ferricyanide dehydrogenase (NFD, ●) and NADH-Q_1 reductase (NQR, ○) activities. The activity is expressed in units/ml. The dotted line denotes the absorbance at 280 nm.

Kinetic constants and their standard errors were calculated according to the method of Cleland [14] as described previously [15].

Protein was determined by the method of Lowry et al. [16] with bovine serum albumin as a standard.

Results

Separation and purification of NADH-quinone reductase (NQR) and NADH-ferricyanide dehydrogenase (NFD)

Details of the purification procedure have been described under Materials and Methods. Table I shows a typical result of purification. Membrane vesicles suspended in 50 mM potassium phosphate (pH 7.5) and 5 mM $MgSO_4$ exhibited the activity of Q_1 reduction for both NADH and d-NADH, but the EDTA-washed membranes lost d-NADH-reactive Q_1 reductase, which

TABLE I

Separation and purification of NADH-quinone reductase (NQR) and NADH-ferricyanide dehydrogenase (NFD) from the membranes of E. coli

Fraction	Protein (mg)	NQR activity for NADH		NFD activity for NADH		NFD activity for d-NADH	
		units	U/mg	units	U/mg	units	U/mg
Membrane vesicles	1000	2380	2.38	3230	3.23	2780	2.78
EDTA-washed membranes	487	1256	2.58	1802	3.70	1271	2.61
$C_{12}E_8$ extracts	255	696	2.73	1346	5.28	852	3.34
DEAE-Sephacel	105	306	2.91	834	7.94	763	7.27
First DEAE-5PW:							
NQR	1.7	57	33.5	–	–	–	–
NFD	5.5	0	0	311	56.5	222	40.4
Second DEAE-5PW (NQR) [a]	0.1	8.7	87	0.7	7.0	0	0
Bio-Gel HTP (NFD)	0.86	0	0	288	335	251	292

[a] The recovery at the peak fraction is shown in this table.

amounted to about 65% of the NADH-Q_1 reductase activity. Thus the recovery of NADH-Q_1 reductase in the EDTA-washed membranes decreased to about half of the original membrane vesicles. On the other hand, the activity of ferricyanide reduction could be measured with both NADH and d-NADH throughout the purifications. Although the enzyme activities were diminished by the EDTA treatment, it was necessary to improve the extraction of enzymes by $C_{12}E_8$ from the membrane vesicles. The $C_{12}E_8$ extracts were chromatographed on DEAE-Sephacel and then on TSK-gel DEAE-5PW. The DEAE-5PW column was developed in the presence of 0.1% Liponox DCH, since the use of $C_{12}E_8$ was ineffective for the separation of enzymes. As shown in Fig. 1, NFD was separated from NQR. NFD was eluted at 50 min (260 mM NaCl) with a minor peak at 60 min, and NQR was eluted at 75 min (320 mM NaCl) with a minor peak at 66 min. After the collection of each major peak fraction, the concentration of Liponox DCH was decreased to about 0.02% to prevent enzyme inactivation. The NQR fraction was rechromatographed on DEAE-5PW (second DEAE-5PW). The NFD fraction was further purified with Bio-Gel HTP in the presence of 10 μM FMN, which was essential for the stabilization of enzyme activity. As calculated on the basis of membrane protein, NQR and NFD were purified 37- and 104-fold, respectively.

On SDS-polyacrylamide gel electrophoresis, NQR still contained several protein bands (Fig. 2B). Among them, a protein band of 47 kDa was concentrated by further purification of NQR with DEAE-5PW. NFD purified with Bio-Gel HTP contained three protein bands after gel filtration (Fig. 2A). Although the purification of NFD to a single protein was difficult at present, NFD was always accompanied by a protein band of 48 kDa.

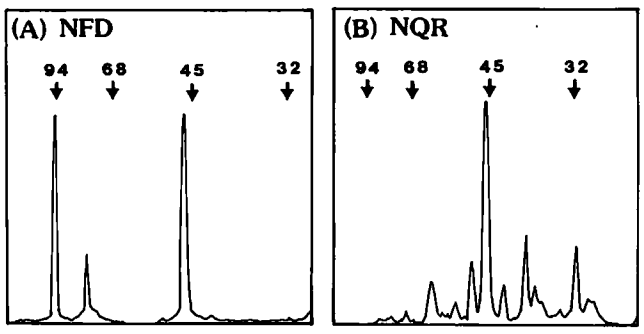

Fig. 2. Densitometer trace of SDS-polyacrylamide gel electrophoresis of NFD (A) and NQR (B) on 8% acrylamide gel. (A) NFD from Bio-Gel HTP after the gel filtration with the specific activity of 498 units/mg protein. (B) NQR from second DEAE-5PW with the specific activity of 87 units/mg protein. Numbers in the figure denote the mass of standard proteins in kDa.

TABLE II

Flavin contents and enzyme activities of NQR and NFD

Enzyme preparation	Flavin (nmol/mg)		Activity (U/mg)
	FAD	FMN	
NQR, 2nd DEAE-5PW	2.97	0.13	82.4
NFD, 1st DEAE-5PW	0.08	2.10	56.5
Bio-Gel HTP [a]	0	12.7	498

[a] After gel filtration.

Flavin contents of NQR and NFD

Table II lists the flavin contents of NQR and NFD together with their enzyme activities. The NQR fraction of the second DEAE-5PW contained about 3 nmol FAD/mg protein. The low flavin content of this preparation may be due to a partial loss of flavin during purifications. In contrast to NQR, NFD contained FMN as a cofactor. Since the addition of FMN was essential for the purification of NFD with Bio-Gel HTP, free FMN in the sample was removed by gel filtration before analysis. This preparation contained 12.7 nmol FMN/mg protein with a specific activity of 498 units/mg protein. Although the NFD fraction of the first DEAE-5PW was not exposed to FMN during purification, it also contained FMN as a major flavin. Thus it is apparent that FMN is a cofactor of NFD.

Properties of the purified NQR and NFD in comparison with the membrane-bound activities

To make clear the functional role of purified enzymes in the respiratory chain, their properties were examined in comparison with those of the membrane-bound activities.

Mg^{2+} and detergent requirements. The purified NQR and NFD required Mg^{2+} for the activities and were saturated at about 5 mM and 20 mM, respectively (Fig. 3A). In addition to Mg^{2+}, NQR required 0.02% Lipo-

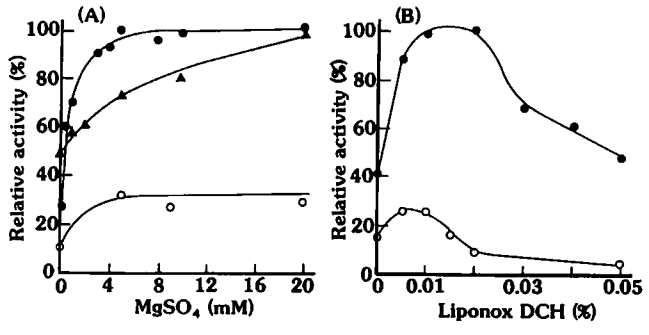

Fig. 3. Effects of Mg^{2+} and Liponox DCH on the activity of NQR and NFD. Enzyme assay was performed under the standard conditions except that the concentration of $MgSO_4$ (A) or Liponox DCH (B) was varied. The activity is expressed in relative activity. (A) NQR assayed in the presence (●) or absence (○) of 0.02% Liponox DCH; NFD assayed in the absence of Liponox DCH (▲). (B) NQR assayed in the presence (●) or absence (○) of 5 mM $MgSO_4$.

nox DCH for optimum activity and higher concentrations of the detergent were inhibitory to the activity (Fig. 3B). When $C_{12}E_8$ was used as detergent, the NQR activity was saturated at 0.02 mM. On the other hand, NFD showed no requirement for the detergent and its activity was unaffected by 0.2% Liponox DCH. Therefore, NQR was assayed in the presence of 5 mM Mg^{2+} and 0.02% Liponox DCH, and NFD in the presence of 20 mM Mg^{2+}, under the standard conditions.

With the membrane vesicles, NQR activity could be measured in the absence of Mg^{2+}, which was stimulated about 1.3-fold with 5 mM Mg^{2+}. The addition of detergent was somewhat inhibitory to the activity. On the other hand, NFD activity of the membrane vesicles was saturated with 20 mM Mg^{2+} with about 50% of the activity in its absence.

Substrate specificities. Table III shows the results of substrate specificities of NQR and NFD. Two enzymes showed a remarkable difference in substrate specificities. NQR could react with NADH but not with d-NADH, whereas NFD could react with both NADH and d-NADH as an electron donor. With respect to electron acceptors, NQR was the most active toward Q_1, whereas NFD could not react with Q_1 at all. The most effective electron acceptor for NFD was ferricyanide. Both enzymes showed a low activity toward menadione and DCIP. These results suggested that d-NADH-reactive NFD purified here is very likely to be a part of d-NADH-reactive oxidase observed with the membrane vesicles [2,3].

Apparent K_m values for NADH and d-NADH. Matsushita et al. [3] reported that *E. coli* GR19N membranes exhibit two apparent K_m values for NADH, that is, 5–15 μM and 50 μM with oxygen or Q_1 as an electron acceptor. These experiments were performed in the reaction mixture containing 50 mM potassium phosphate (pH 7.5) and 5 mM $MgSO_4$. To confirm this, the kinetic properties of the membrane vesicles of *E. coli* C600 were determined under the same conditions. NADH-Q_1 activity of the membrane vesicles exhibited two K_m values for NADH, which were calculated to be 19.1 ± 4.2 and 43.0 ± 2.0 μM, respectively. When d-NADH was used as an electron donor, the K_m values for d-NADH measured with Q_1 and ferricyanide were 21.7 ± 0.7 and 19.9 ± 1.6 μM, respectively. These values coincided well with the low K_m value for NADH. Apparently, there are two reactive sites for NADH in the membranes: the high-affinity site can react with d-NADH as well as NADH and the low-affinity site reacts exclusively with NADH.

TABLE III

Substrate specificities of NQR and NFD

The enzyme activities were assayed under the standard conditions except that electron donor and acceptor were varied as described below and are expressed in units/mg protein.

Enzyme	Electron donor	Electron acceptor			
		Q_1	ferricyanide	menadione	DCIP [a]
NQR	NADH	82.4	6.66	9.25	1.93
NFD	NADH	0	335	7.68	24.9
	d-NADH	0	292	8.23	18.6

[a] Dichlorophenolindophenol.

TABLE IV

Apparent K_m values of NQR and NFD for electron donors

The reaction mixture contained 50 mM potassium phosphate (pH 7.5), 5 mM $MgSO_4$, 1.0 mM ferricyanide or 8 μM Q_1 and varied concentrations of NADH or d-NADH. For NQR assay, 0.02% Liponox DCH was added to the reaction mixture. The activation of NQR was performed by preincubating the enzyme with 0.1 mM NADH for 2 min at 30 °C and then it was used for assay.

Enzyme	Reaction measured	K_m (μM)	V (U/mg)
NQR	NADH-Q_1	96.5 ± 12.7	77.8 ± 7.8
	NADH-ferri.	45.2 ± 9.2	8.1 ± 0.7
NQR [a]	NADH-Q_1	48.3 ± 3.2	123 ± 4.1
	NADH-ferri.	43.8 ± 8.9	26.4 ± 2.5
NFD	NADH-ferri.	7.3 ± 0.9	222 ± 8.4
	d-NADH-ferri.	8.8 ± 1.0	185 ± 7.1

[a] The enzyme was activated before assay.

Table IV is the results of K_m values for NADH or d-NADH of the purified NQR and NFD. The K_m value of NQR for NADH was 96.5 μM with Q_1 as an electron acceptor. As will be shown later, NQR was activated by the preincubation with NADH. With the activated NQR, the K_m value for NADH decreased to 48.3 μM. The K_m for NADH measured with ferricyanide was about 45 μM and it was unaffected by the preincubation treatment. These K_m values were very similar to that of the low-affinity site in the membranes. The maximum velocities of NQR for Q_1 and ferricyanide were increased by the preincubation with NADH.

In contrast to NQR, the K_m values of NFD for NADH and d-NADH were very low amounting to 7–9 μM. Although this value was lower than that measured with the membrane vesicles, NFD is very likely to correspond to the high affinity site of the membrane vesicles that reacts with both NADH and d-NADH.

Effects of preincubation with electron donor. Gutman et al. [17] first reported that NFD activity extracted from freeze-dried membranes of *E. coli* is very unstable to pretreatment with NADH. Matsushita et al. [3] also demonstrated that d-NADH-Q_1 activity of membrane vesicles of *E. coli* decreases by preincubation with d-NADH. As shown in Fig. 4A, NFD was found to be very sensitive to the preincubation with d-NADH and NADH; the former being more effective than the latter. On the other hand, NQR was stable and rather activated by preincubation with NADH (Fig. 4B). NQR, how-

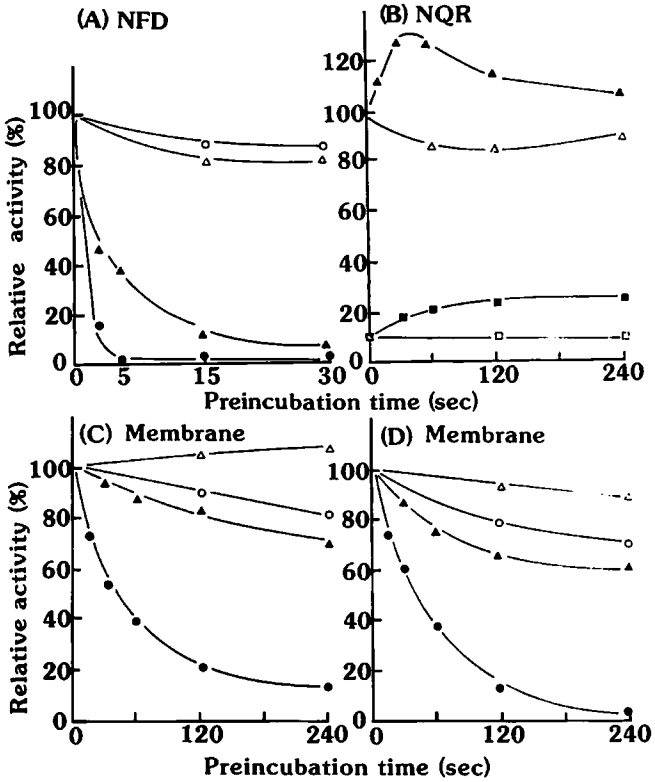

Fig. 4. Effects of preincubation with electron donor on the enzyme activities. NFD (A), NQR (B) or the membrane vesicles (C and D) were preincubated at 30 °C in the presence of 0.2 mM NADH (▲) or d-NADH (●) for indicated time in the absence of electron acceptor and then the reaction was started by the addition of 1.0 mM ferricyanide (A and C) or 8 μM Q_1 (B and D). In (B), the activity toward ferricyanide (■) was also measured. Open symbols represent the activities obtained by the preincubation without electron donor as controls. Since the presence of FMN partly prevented the inactivation of NFD, the NFD fraction from first DEAE-5PW was used in this experiment. The concentrations of enzymes used are as follows: NFD, 4 μg/ml; NQR, 0.2 μg/ml; the membrane vesicles in (C), 50 μg/ml; and in (D), 6.3 μg/ml.

ever, was not activated by the preincubation with d-NADH.

With the membrane vesicles, the membrane-bound d-NADH-ferricyanide activity was also very sensitive to d-NADH preincubation (Fig. 4C). Although the rate of inactivation was slower than that of NFD, more than 80% of the activity was lost in 240 s. These results suggested that the d-NADH-reactive NFD in the membranes is inactivated by the preincubation with d-NADH. When the membrane vesicles were preincubated with NADH, only about 30% of NADH-ferricyanide activity was lost in 240 s. The activity of membrane vesicles for d-NADH (2.3 units/mg protein) corresponded to about 80% of that for NADH (2.9 units/mg protein). Since the d-NADH-reactive site in the membranes reacted with NADH also, its inactivation should lead to a more pronounced decrease in the activity for NADH. However, it was found that the activity of NQR for ferricyanide increases about 3-fold by the preincubation with NADH (Table IV and Fig. 4B). Supposing that the d-NADH-reactive site was inactivated by the NADH preincubation similar to NFD, the moderate decrease in the NADH-ferricyanide activity could be explained by the activation of NADH-ferricyanide activity of NQR in the membranes. In a separate experiment, it was confirmed that d-NADH-ferricyanide activity was indeed inactivated by the NADH preincubation.

Similar to the activities toward ferricyanide, d-NADH-Q_1 activity was markedly inactivated, whereas the NADH-Q_1 activity moderately decreased, as shown in Fig. 4D. The membrane vesicles used in this experiment exhibited d-NADH-Q_1 activity (1.7 units/mg) corresponding to 65% of NADH-Q_1 activity (2.6 units/mg). Since NQR was activated by the NADH preincubation (Fig. 4B), the moderate decrease in NADH-Q_1 activity could be explained by the inactivation of d-NADH-reactive site. These results strongly suggested that d-NADH-reactive NFD purified here is a component of d-NADH-Q_1 reductase in the membranes and thus the latter is sensitive to the preincubation with d-NADH and NADH.

Generation of membrane potential in the inverted membrane vesicles

The generation of $\Delta\psi$, positive inside, was monitored by the absorbance band shift of oxonol VI. As shown in

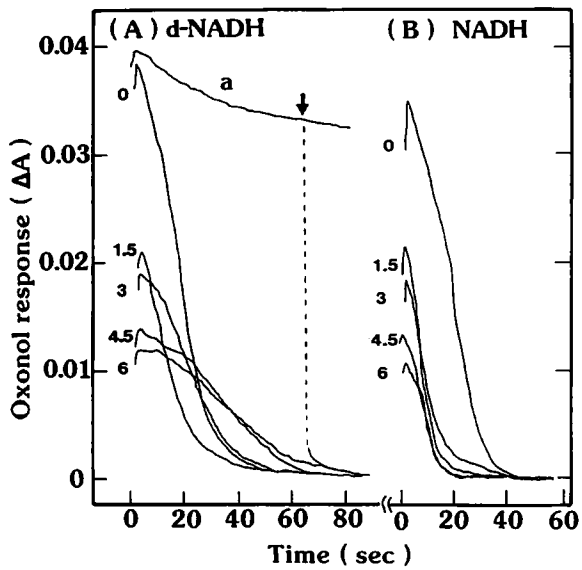

Fig. 5. Generation of $\Delta\psi$ in the inverted membrane vesicles. Experimental conditions were described under Materials and Methods, and the oxonol response was monitored at 625–587 nm. In curve a, 10 mM KCN and 10 μM Q_1 were omitted from the reaction mixture and the d-NADH oxidase reaction was started by the addition of 0.1 mM d-NADH at 20 °C. At the arrow, 2 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was added to the reaction mixture. The numbers on each curve denote the time of preincubation in min with d-NADH (A) or NADH (B) in the absence of Q_1. After the preincubation, d-NADH-Q_1 (A) and NADH-Q_1 (B) reactions were started by the addition of Q_1 at zero time.

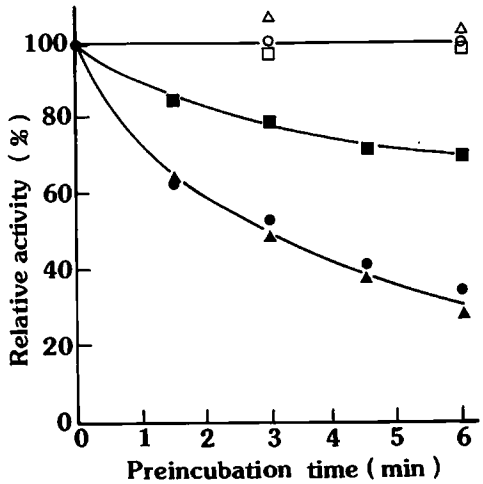

Fig. 6. Time-courses of the decrease in oxonol response and the enzyme activities. The membrane vesicles were preincubated with d-NADH for indicated times under the conditions same as in Fig. 5, and then the oxonol response (●), d-NADH-Q_1 (▲) and NADH-Q_1 (■) activities were determined. Results are expressed in relative activity. Open symbols represent the results obtained by the preincubation without d-NADH as controls.

Fig. 5A, the oxidation of d-NADH by oxygen (curve a) induced the oxonol response and it continued more than 3 min until d-NADH was exhausted. Although less effective, NADH also gave a result similar to d-NADH. The $\Delta\psi$ was completely collapsed by the addition of 2 μM CCCP.

In the presence of 10 mM KCN, a large magnitude of $\Delta\psi$ was generated by d-NADH-Q_1 (Fig. 5A) and NADH-Q_1 (Fig. 5B) reactions without preincubation. Due to the presence of high concentration of membrane vesicles (100 μg/ml), 10 μM of Q_1 was quickly reduced and the $\Delta\psi$ was dissipated with the consumption of Q_1. Since the d-NADH-reactive site of the membrane vesicles was specifically inactivated by the preincubation with electron donors (Fig. 4D), the membrane vesicles were preincubated with d-NADH or NADH and then the $\Delta\psi$ was determined (Fig. 5A and B). In both cases, the magnitude of $\Delta\psi$ decreased to the same extent with the increase in the preincubation time. Fig. 6 shows the time courses of decrease in the $\Delta\psi$ together with the enzyme activities. Since these experiments were performed at 20°C, the rate of inactivation of d-NADH-Q_1 activity was slower than that shown in Fig. 4D. But its inactivation proceeded in parallel with that of the decrease in $\Delta\psi$. After preincubation for 6 min, the membrane vesicles retained about 30% of d-NADH-Q_1 activity, exhibiting about 30% of the maximum oxonol response. Under those conditions, more than 70% of NADH-Q_1 activity was maintained. These results indicated that d-NADH-reactive Q_1 reductase is directly coupled to the generation of $\Delta\psi$ and that the reduction of Q_1 by the enzyme that reacts exclusively with NADH and is rather activated by the preincubation treatment (Fig. 4B) has no capacity to generate $\Delta\psi$. As shown in Fig. 5, the $\Delta\psi$ generated by d-NADH lasted longer than that by NADH after the partial inactivation of d-NADH-Q_1 activity. Since d-NADH was oxidized only at the d-NADH-reactive site in the membranes, these results also support the above notion. Although not shown here, no appreciable magnitude of $\Delta\psi$ was generated by the reduction of ferricyanide with either d-NADH or NADH.

Discussion

The FMN-containing NFD purified in this paper reacts with both NADH and d-NADH (Table III), has a high affinity for these electron donors (Table IV), and is very sensitive to preincubation with the electron donors (Fig. 4A). These properties are more or less the same as those observed with a d-NADH-reactive quinone reductase of the membrane vesicles (Fig. 4D), which generates an inside-positive $\Delta\psi$ by the reduction of Q_1 (Fig. 5A). On the other hand, the FAD-containing NQR reacts only with NADH with a low affinity for NADH (Tables III and IV). With the membrane vesicles, a quinone reductase reacting with only NADH has no capacity to generate $\Delta\psi$ (Fig. 5 and 6). Thus the respiratory chain-linked NADH-quinone reductases of *E. coli* can be formulated as shown in Fig. 7.

The two types of membrane-bound NQR are termed NQR-1 and NQR-2, which correspond to NADH dh-I and NADH dh-II, respectively, named by Ohnishi et al. [2] and Matsushita et al. [3]. The type 1 is the energy-generating NADH-quinone reductase. According to this definition, NQR purified here corresponds to NQR-2 and NFD is a part of NQR-1. Although NFD is not able to reduce Q_1 by itself, Q_1 is reduced to ubiquinol by d-NADH with the membrane vesicles. Thus, in addition to NFD, other components are required for the reduction of Q_1, which are very likely to be iron-sulfur clusters identified by Ohnishi et al. [2,18]. Since the addition of ferricyanide to the membrane vesicles generated no significant $\Delta\psi$, the site(s) of energy coupling should be located on the reaction after NFD and thus on the iron-sulfur clusters.

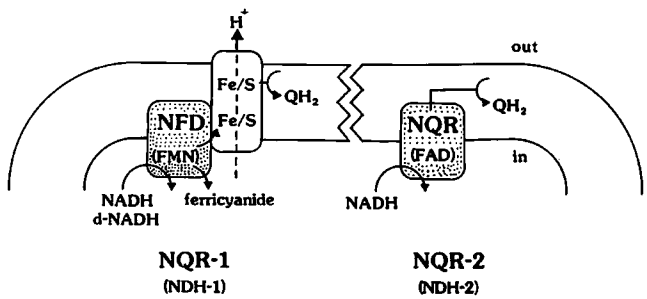

Fig. 7. Two types of NADH-quinone reductase in the respiratory chain of *E. coli*.

With respect to NQR, its properties are identical to that of the enzyme purified by Jaworowski et al. [6] and it corresponds to NQR-2 in Fig. 7. The physiological role of NQR-2, however, is not clear at present. But since the NQR-2 of *E. coli* has low affinity for NADH, this enzyme will become functional when the concentration of NADH increases to a high level. Thus, NQR-2 is likely to participate in the regulation of NADH level in the cytoplasm without being much influenced by $\Delta\tilde{\mu}_{H^+}$.

Gutman et al. [17] extracted NADH-ferricyanide dehydrogenase from freeze-dried membranes of *E. coli* without using any detergent. Although the extracted enzyme was not further purified, the enzyme was rapidly inactivated by the incubation with NADH in the absence of electron acceptor. Thus the preparation of Gutman et al. is likely to contain NFD as a major constituent. Although they suggested the conversion of enzyme from a high-affinity form I to a low-affinity form II for NADH by the preincubation with NADH, their results can be explained by the presence of both NFD and NQR in their preparation. This explanation is also in accordance with the analytical data on the presence of both FMN and FAD in their preparation.

Recently Yagi et al. [19] purified two types of NQR from *Thermus thermophilus* HB-8. One of these (NDH-1) is a complex composed of ten different polypeptides and the other (NDH-2) is a single polypeptide of 53 kDa (SDS-polyacrylamide gel electrophoresis). NDH-1 contains FMN, non-heme iron and acid-labile sulfide, whereas NDH-2 contains FAD and no iron. The former was presumed to contain an energy-coupling site. Thus, although both enzymes reacted with NADH and d-NADH, the presence of two types of NQR seems to be general in bacterial respiratory chain.

With the membrane vesicles of *E. coli*, d-NADH-Q_1 reductase was very unstable for various treatments such as simple washing with a buffer, or the preincubation with EDTA or d-NADH, suggesting that the iron-sulfur clusters were more fragile than NFD. Thus the reconstitution of energy-generating d-NADH-Q_1 reductase from the purified NFD and NFD-deprived membranes has so far been unsuccessful. Very recently, we succeeded in isolating d-NADH-reactive Q_1 reductase as a whole complex from the membranes. The purification and analysis of the complex will provide detailed information on the structure and the reaction mechanism of energy-generating NADH-quinone reductase complex of *E. coli*.

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