Jour. Microbiol. June 1996 p. 206-213

Vol. 34, No. 2

An FMN-Containing NADH-Quinone Reductase from *Streptomyces* sp.

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(Received December 20, 1995/Accepted March 13, 1996)

NADH-quinone reductase was purified 22-fold from the cytosolic fraction of *Streptomyces* sp. IMSNU-1 to apparent homogeneity, with an overall yield of 9%, by the purification procedure consisting of ammonium sulfate precipitation and DEAE Sephacel, Sephacryl S-200 and DEAE 5 PW chromatographies. The molecular mass of the enzyme determined by gel filtration chromatography was found to be 110 kDa. SDS-PAGE revealed that the enzyme consists of two subunits with a molecular mass of 54 kDa. The enzyme contained 1 mol of FMN per subunit as a cofactor. The A_{272}/A_{457} ratio was 6.14 and the molar extinction coefficients were calculated to be 20,800 and 25,400 $M^{-1}cm^{-1}$ at 349 and 457 nm, respectively. The *N*-terminal sequence of the enzyme contained the highly conserved fingerprint of ADP-binding domain. The enzyme used NADH as an electron donor and various quinones as electron acceptors. Cytochrome *c* was practically inactive. Air-stable flavin semiquinone was produced by the addition of NADH to the enzyme. Also, naphthosemiquinone was detected in the reaction mixture containing the enzyme.

Key words: NADH-quinone reductase, FMN, semiquinone, *Streptomyces* sp

Several flavoprotein oxidoreductases have been known to be able to catalyze the reduction of quinone compounds; mitochondrial NADH-ubiquinone oxidoreductase from yeast [1], dihydrolipoamide dehydrogenase [2], DT-diaphorase [3], NADPH-cytochrome *c* reductase from yeast [4], and membrane bound NADPH-cytochrome P 450 reductase [5]. Flavoprotein oxidoreductases catalyze either oneor two-electron reduction: DT-diaphorase catalyzes two-electron reduction, while NAD(P)H-cytochrome P450 reductase catalyzes single-electron reduction [6]. The single-electron reduction of quinones by NAD(P)H-cytochrome P450 reductase can lead to the formation of semiquinones that are readily autoxidized with the formation of superoxide anion radical. DT-diaphorase has been proposed to play an important role in the detoxification of quinones. This enzyme can catalyze two-electron transfer to several quinones with the formation of relatively stable hydroquinones [7]. Dihydrolipoamide dehydrogenase is known to be a member of FAD-cystine oxidoreductases including glutathione reductase [8] and thioredoxin reductase [9]. These enzymes catalyzes two-electron transfer between the oxidized state (E) and the reduced state (EH_2).

Streptomyces are highly aerobic microorganisms and have distinct catalase-peroxidase [10] and lack glutathione-glutathione reductase systems [11]. In the present paper, we report some properties of a NADH-quinone reductase purified from *Streptomyces* sp. IMSNU-1.

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Materials and Methods

Chemicals

NADH, NADPH, FAD, FMN, riboflavin, 2,6-dichlorophenolindophenol, 5,5'-dithiobis-(2-nitroben zoic acid) (DTNB), 1,4-benzoquinone, 2-hydroxy-1,4-naphthoquinone, methyl-1,4-benzoquinone, 2-methyl-1,4-naphthoquinone, 1,4-naphthoquinone, cytochrome *c*, DEAE Sephacel, Superose 12 prep grade, bovine glutathione reductase, lipoamide dehydrogenase from *Clostridium kluyveri*, and porcine cytochrome *c* reductase were purchased from Sigma, Pro-

tein-Pak DEAE 5PW from Waters, and molecular mass markers for gel filtration and SDS-PAGE from Boehringer Mannheim. All other reagents used were of the highest quality generally available.

Microorganism and culture conditions

Streptomyces sp. IMSNU-1 was cultivated under the same conditions as described previously by Youn *et al.* [10].

Enzyme assay

The activity of NADH-quinone reductase was determined spectrophotometrically at 37℃ by measuring the decrease of absorbance at 340 nm with 0.3 mM quinone and 0.2 mM NAD(P)H in 50 mM sodium phosphate buffer (pH 7.4). One unit (U) of enzyme activity was defined as the amount of enzyme which converted 1 mol of NAD(P)H per min according to the method proposed by Buswell *et al.* [12].

Protein content

The protein concentration was determined according to the method proposed by Bradford [13]. The purity of protein was estimated by Imaging Densitometer (Model GS-670, Bio-Rad).

Enzyme purification

The mycelia, cultivated aerobically in YEME medium (0.5% yeast extract, 0.5% casamino acid, 0.5% peptone, 0.3% malt extract, and 1% dextrose, pH 7.0), were harvested by aspiration on filter paper in a Büchner funnel and washed with 0.85% KCl solution. The washed mycelia were suspended in 20 mM sodium phosphate buffer (pH 7.4), and broken with a bead-beater (Biospec. Production) for 5 min, and the homogenate was centrifuged at 4,000×g for 15 min. Solid ammonium sulfate was added to the supernatant up to 80% saturation. The precipitate was collected by centrifugation at 12,000×g for 20 min and resuspended in the same buffer. The resuspended solution was dialyzed against the same buffer overnight. The dialyzate was loaded onto a DEAE Sephacel (4×40 cm) previously equilibrated with the same buffer and then proteins were eluted with a linear gradient of 0~0.8 M NaCl in the same buffer. The active fractions were pooled and desalted with an Amicon YM 10 membrane. The desalted fraction was applied to Sephacryl S-200 HR (2.8×110 cm) column and eluted with the same buffer. The active fractions were collected and concentrated with an Amicon YM 10 membrane again. The concentrated sample was purified by Waters Delta Prep 4000 chromatography system: the sample was loaded onto a Protein-Pak DEAE 5PW column (2.15 ×15 cm) equilibrated with the same buffer. After the column was washed with 0.2 M NaCl in the same buffer, proteins were eluted with a linear gradient of 0.2~0.5 M NaCl in the same buffer. The active fractions were again pooled and exchanged with the same buffer and stored at -70℃.

Molecular mass determination

The molecular mass of the purified enzyme was determined by gel filtration chromatography on Superose 12 prep grade (2×60 cm). The column was calibrated with catalase (240 kDa), aldolase (158 kDa), albumin (68 kDa), and chymotrypsinogen (25 kDa). For the cross-linked proteins, dimethyl suberimidate was used as a cross-linking reagent according to the method proposed by Davis and Stark [14]. Cross-linked proteins were electrophoresed on 7.5% SDS-PAGE according to the method proposed by Laemmli [15]. As standard markers, α_2-macroglobulin (170 kDa), (β-galactosidase (116 kDa), fructose-6-phosphate kinase (85 kDa), glutamate dehydrogenase (55 kDa), and aldolase (39 kDa) were used. The gel was silver-stained after electrophoresis according to the method proposed by Morrissey [16].

N-terminal sequence analysis

The purified enzyme was subject to SDS-PAGE. Electrotransfer of proteins was carried out according to the method proposed by Towbin *et al.* [17]. Analysis of the *N*-terminal sequence was carried out by automated Edman degradation with a MilliGen/Biosearch 6600 Prosequencer system (Millipore).

Flavin analysis

The purified enzyme (0.5 mg protein) was precipitated with 5% TCA solution and then kept on ice for 30 min. The sample was centrifuged at 12,000×g for 15 min to discard the precipitated protein; the yellow supernatant was adjusted to pH 7.0 with 1 N NaOH and concentrated with a Speed-Vac concentrator (Savant). The concentrated sample was analyzed by chromatography on thin-layer silica gel plates (Merck). These samples were developed with the mixed solution of butanol-acetic acid-water (12 : 3 : 5) according to the method proposed by Poole and Claiborne [18].

Spectroscopic studies

The absorption spectra of all samples were measured with a Shimadzu UV-265 spectrophotometer. Spectra were obtained at 25℃ using a 1-cm quartz cuvette containing native or reduced enzyme dissolved in 50 mM sodium phosphate buffer (pH 7.4). To confirm the production of flavin semiquinone radical of the enzyme, 40

Table 1. Purification step of NADH-quinone reductase from *Streptomyces* sp. IMSNU-1. One unit of enzyme activity was defined as the amount of enzyme which converted 1 μmol of NADH per min according to the method proposed by Buswell *et al.* [12]

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Cride extract	1743.7	54.5	0.03	100	1
Ammonium sulfate precipitation	779.0	42.1	0.05	77.2	1.7
DEAE Sephacel charomatogaphy	73.8	15.5	0.21	28.4	6.8
Sephacryl S-200 charomatographay	20.2	6.2	0.31	11.4	9.9
DEAE 5 PW charomatography	7.3	5.0	0.69	9.2	22.1

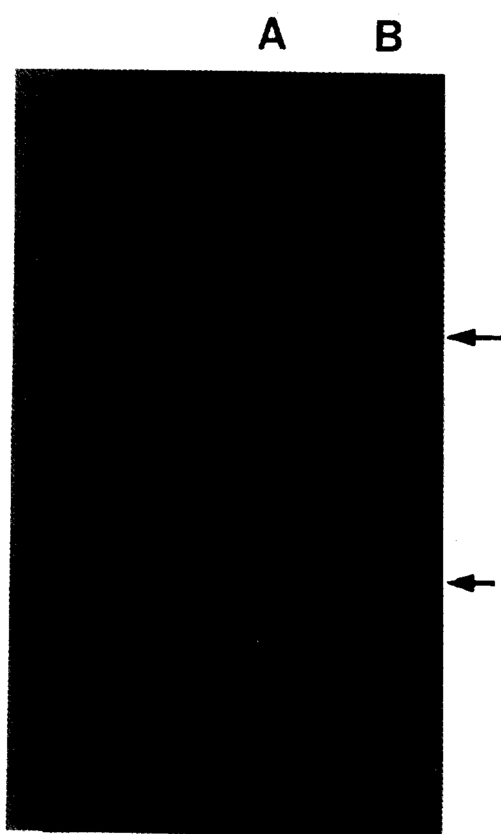

Fig. 1. 7.5% SDS-PAGE of NADH-quinone reductase from *Streptomyces* sp. IMSNU-1. (A) native enzyme (0.5 μg) and (B) cross-linked enzyme (0.5 μg) were loaded into each lane. The gel was calibrated with α2-macroglobulin (170 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (85 kDa), glutamate dehydrogenase (55 kDa) and aldolase (39 kDa).

μM NADH was added to the solution containing 18 μM native enzyme under aerobic conditions.

The fluorescence spectra of NADH-quinone reductase were obtained by means of an Aminco 48000$_{TM}$S spectrofluorometer (SLM Instruments). The excitation spectra were measured at 521 nm-emission and the emission spectra were obtained at 470 nm-excitation.

EPR studies were carried out by means of a Bruker ESP 300S EPR spectrometer under the following conditions; 9.77 GHz of microwave frequency, 10 mW of microwave power, and 1.0 mT of modulation amplitude.

Results and Discussion

Enzyme purification

The NADH-quinone reductase was purified 22-fold relative to the cell extract with a recovery of 9% as summarized in Table 1. The purity of the final preparation was estimated to be approximately 85% on silver-stained SDS-polyacrylamide gel by means of densitometry (Fig. 1).

Molecular Weight

The molecular mass of the enzyme was determined to be 54 kDa after SDS-PAGE in the presence or absence of β-mercaptoethanol. To obtain an insight into the subunit composition, the enzyme was incubated with a cross-linking agent, dimethyl suberimidate, prior to denaturation and electrophoresis. Two bands (54 and 110 kDa) were visualized after silver-staining (Fig. 1). The native enzyme has a molecular mass of 110 kDa as estimated by gel filtration chromatography on Superose 12 prep grade (2×60 cm). This enzyme is, therefore, considered to be composed of two subunits which are not covalently cross-linked.

Prosthetic group

The absorption spectra of NADH-quinone reductase revealed peaks at 272, 349 and 457 nm, and the A_{272}/A_{457} ratio was 6.14 for the enzyme (Fig. 4). The molar ex-

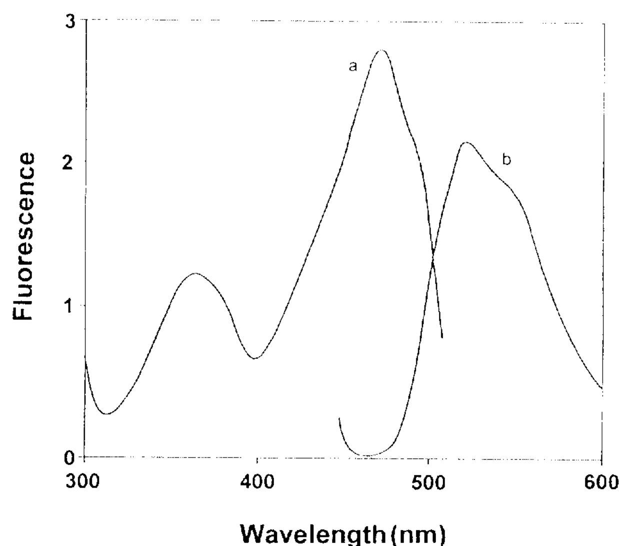

Fig. 2. The fluorescence spectra of NADH-quinone reductase. The excitation spectra (a) were measured at 521 nm-emission *vice versa* the emission spectra (b) were obtained at 470 nm-excitation.

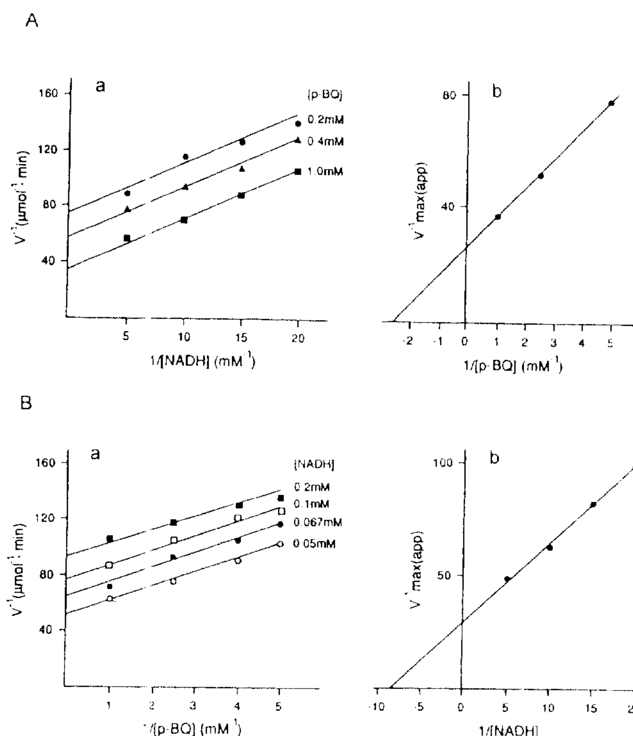

Fig. 3. Determination of the steady-state kinetic parameters of *p*-benzoquinone and NADH. (A) Lineweaver-Burk plot *versus* the concentration of NADH and the intercepts from (a) are plotted against the inverse concentrations of *p*-benzoquinone (b). (B) Lineweaver-Burk plot *versus* the concentration of of *p*-benzoquinone and the intercepts from (a) are plotted against the inverse concentration of NADH (b).

tinction coefficients were calculated to be 20,800 and 25,400 $M^{-1}cm^{-1}$ at 349 and 457 nm, respectively. The fluorescence spectra of the enzyme is also presented in Fig. 2. Both excitation and emission spectra showed spectra typical of flavoproteins.

The addition of 5% TCA to the enzyme solution resulted in the dissociation of flavin from the enzyme, indicating that flavin was noncovalently associated with the enzyme. The mobility of the TCA-extract was nearly equal to that of FMN and clearly distinct from those of riboflavin and FAD. The FMN content was calculated to be 1 mol FMN per subunit on the basis of 54 kDa of the molecular mass and the molar extinction coefficient of FMN (ε_{445}=12,500 $M^{-1}cm^{-1}$) [19].

DT-diaphorase from rats has 1 mol of FAD per 55 kDa-subunit [3], while microsomal NADPH-cytochrome P450 reductase from liver is known to contain 1 mol of FMN and 1 mol of FAD per 76 kDa-polypeptide chain [5]. On the other hand, NAD(P)H-cytochrome *c* reductase from *Saccharomyces cerevisiae* contains 0.63 mol of FMN per 34 kDa-subunit [20]. NAD(P)H-cytochrome P450 reductase from *Saccharopolyspora erythraea* contains FAD as a cofactor [21]. In addition, all dihydrolipoamide dehydrogenasea are known to be members of the FAD-containing flavoproteins.

Kinetic proterties

The kinetic mechanism of NADH-quinone reductase was investigated using initial velocities. In an experiment with varying *p*-benzoquinone concentrations in the presence of four different fixed NADH concentrations, the double reciprocal plot showed parallel lines, indicating a strong evidence of a ping-pong mechanism. The secondary plot of the reciprocal of intercepts (V^{-1}max(app)) versus the reciprocal of p-benzoquinone concentrations revealed that the K_m value for *p*-benzoquinone was 2.4 M (Fig. 3A). The double reciprocal plot obtained from an experiment with varied NADH concentrations and three different fixed *p*-benzoquinone concentrations showed parallel lines, which provided a strong evidence of a ping-pong mechanism. The secondary plot of the reciprocal of intercepts (V^{-1}max(app)) against the reciprocal of NADH concentrations revealed that K_m value for NADH was 119 μM (Fig. 3B).

Substrate specificity

The NADH-quinone reductase mainly used NADH as an electron donor, although it could use NADPH at a reduced rate (Table 2). As electron acceptors, 2,6-dichlorophenolindophenol and p-benzoquinone were the most active, but cytochrome *c* was practically inactive. On the other hand, NAD(P)H-cytochrome P450 reductase from *Saccharopolyspora erythraea* was reported to ex-

Table 2. Substrate specificity of NADH-quinone reductase from *Streptomyces* sp.

Substrate[a]	Specific activity[b]	
	NADH oxidation (μmol/min/mg of protein)	NADPH oxidation (μmol/min/mg of protein)
1,4-benzoquinone	35.56	9.11
methyl-1,4-benzoquinone	25.40	1.28
1,4-naphthoquinone	14.68	0.48
2-methyl-1,4-naphthoquinone (menadione)	7.18	0.11
2-hydroxy-1,4-naphthoquinone (lawsone)	1.95	1.40
potassium ferricyanide	7.87	1.71
2,6-dichlorophenolindophenol	61.40	2.25
cytocrome *c*	0.80	0
lipoic acid	15.60	0.11
lipoamide	16.30	0.15
glutathione	0.18	0
cystine	0.80	0
thioredoxin	0.20	0
DTNB[c]	0.49	0
oxygen[d]	0	0

[a] The reaction mixture contained 0.3 mM substrates (in case of cytochrome *c*, 50 μM; thioredoxin, 40 μM) and 0.2 mM NAD(P)H in 50 mM sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of the enzyme (1.5 μg) to the reaction mixtures at 37℃.

[b] Specific activites were determined from the rate of decrease at 340 nm and expressed as NAD(P)H mmol/min/mg of protein. The molar extinction coefficient of NADH was 6,220 $M^{-1}cm^{-1}$.

[c] Specific activity was determined from the increase at 412 nm. The molar extinction coefficient of DTNB was 13,600 $M^{-1}cm^{-1}$ [22].

[d] The reaction buffer was bubbled with oxygen gas for 10 min, and the NAD(P)H was added to the final concentration of 0.2 mM. The other conditions are the same as described above.

hibit a significant amount of the reduction of cytochrome *c* [21]. Lipoic acid and lipoamide could be used as substrates, but other sulfur compounds such as glutathione, thioredoxin, cystine, and DTNB were not used. Among the quinone compounds, benzo-forms were more active than naphtho-forms, and quinones without side-chains were more active than those with side-chains. Methylation in the C-2 position of the naphthoquinone ring had less effect on the activity than hydroxylation in C-2 position. The enzyme was also able to reduce potassium fericyanide. The enzyme had no detectable NADH oxidase activity under oxygen-saturated conditions. The substrate specificity of the enzyme was similar to that of cytosolic DT-diaphorase except that the enzyme could not use NADPH as an electron donor.

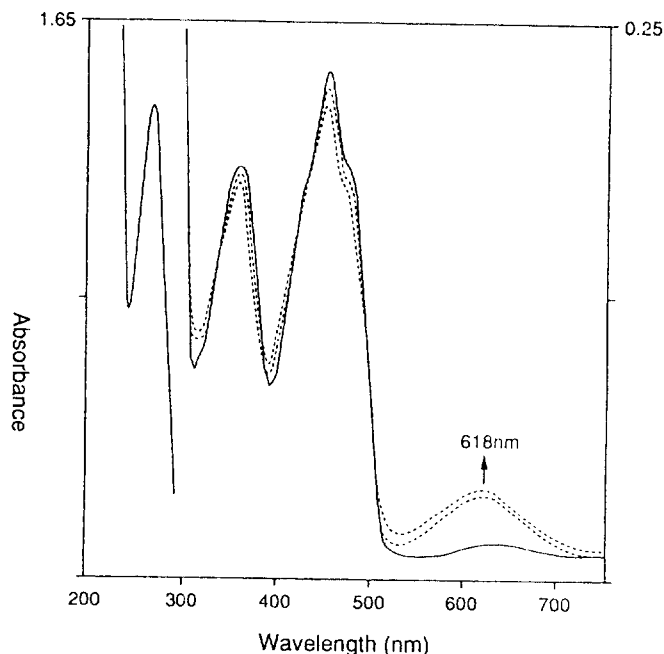

Fig. 4. The formation of air-stable flavin semiquinone intermediate of NADH-quinone reductase. Purified enzyme (18 μM) was dissolved in 50 mM phosphate buffer (pH 7.4). The reduced form (---) was obtained by the addition of 40 μM NADH to the native enzyme (—). The scanning interval was 2 min.

Air-oxidation of the enzyme

The spectral changes of the NADH-quinone reductase following the addition of 40 μM NADH under aerobic conditions are presented in Fig. 4. Within 2 min after the addition of NADH, a new absorbance maximum at 618 nm appeared, indicating an air-stable flavin semiquinone intermediate that was revealed to be an one-electron reduced species. In NAD(P)H-cytochrome *c* reductase from yeast, anaerobic titrations with NAD(P)H revealed two-electron reduction of FMN, with no spectrally observable flavin semiquinone intermediates [20]. FMN-depleted, NAD(P)H-cytochrome P450 reductase exhibited the absorption maxima of flavin semiquinone at 592 nm [23].

Production of naphthosemiquinone radical

As shown in Fig. 4, the enzyme produced an air-stable flavin semiquinone with NADH as an electron donor, indicating that naphthosemiquinones can be produced by one-electron transfer from flavin semiquinone to oxidized form of quinone. Using EPR spectroscopy, Naphthosemiquinone radical ($a_{H(2)}=a_{H(3)}=0.32$ mT, $a_{H(5)}=a_{H(8)}=0.63$ mT, $a_{H(6)}=a_{H(7)}=0.66$ mT) was detected in the reaction mixture by the action of the enzyme (Fig. 5). It has been reported that the nonenzymatic reaction of naphthoquinone with NAD(P)H resulted in the formation

of semiquinone radical in the limited conditions, e. g., in higher concentration (0.5~3 mM) of NAD(P)H and in boric acid-borax buffer (pH 9.0). On the other hand, little was detected in lower concentration of NAD(P)H and phosphate buffer (pH 7.4) [24]. In fact, naphthosemiquinone was not detected at all with 0.1 mM NADH in 50 mM sodium phosphate buffer (pH 7.4). Naphthosemiquinones are known to produce superoxide anion radical by reacting with oxygen molecule [25]. It has been reported that NAD(P)H-cytochrome P450 reductase could generate superoxide anion radical. NADPH-ferredoxin oxidoreductase was recently identified as a paraquat diaphorase capable of generating superoxide anion radical in *Escherichia coli* [26].

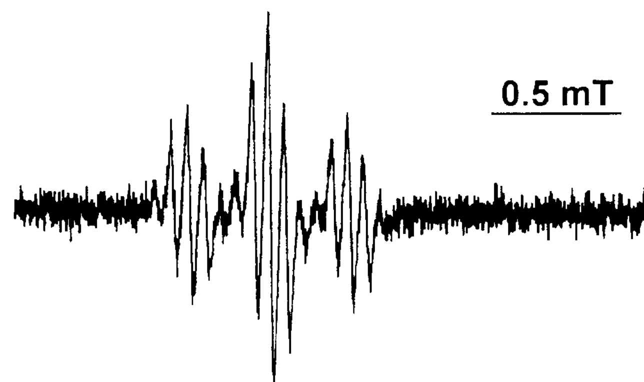

Fig. 5. EPR spectra after the reaction of 1,4-naphthoquinone. The reaction mixtures contained 1 mM 1,4-naphthoquinone, 0.1 mM NADH in 50 mM sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 2.5 μg of the enzyme. The EPR spectra was obtained at room temperature under the following condition; 9.77 GHz of microwave frequency, 10 mW of microwave power, and 1.0 mT of modulation amplitude.

Putative NADH and FMN binding site

The *N*-terminal sequence of 25 amino acid residues of NADH-quinone reductase from *Streptomyces* is presented in Fig. 6 along with those of other flavoproteins. These sequences have the highest homology with that of dihydrolipoamide dehydrogenase from *Clostridium sporogenes* (identities, 58%; similarities, 88%) and contains the highly conserved fingerprint of the ADP-binding site found in many other NAD(H)- or FAD-binding proteins. The sequences of ADP-binding site reveal universally conserved Gly-Xaa-Gly-Xaa-Xaa-Gly sequences which can fold in a βαβ substructure allowing the formation of a pocket around the coenzyme [27]. These conserved sequences were mainly found to be located close to the *N*-terminal amino acid sequence of NAD(H)- or FAD-binding proteins. In FAD-containing flavoproteins, it has been speculative whether these regions are necessary for binding of the ADP-moiety of FAD or NAD(H). Fortunately, the NADH-quinone reductase from *Streptomyces*

Enzyme	Sources	Sequence
NADH-quinone R	*Streptomyces*	1-A N D A S T V F D L V I L **G** G **G** S G **G** Y A A **A** L R
Dihydrolipoamide DHase	*C. sporogenes*	1-A D E I F D L I V L **G** A **G** P G **G** Y V G **A** I R
Sulfide-quinone R	*O. limnetica*	1-A H V A V I **G** A **G** L G **G** L P T **A** Y E
Glutathione R	*E. coli*	1-M T K H Y D Y I A I **G** G **G** S G **G** I A S I N R
NADH DHase	*E. coli*	1-T M T P L K K I V I V **G** G **G** A G **G** L E M **A** T Q
Rubredoxin R	*P. oleovorans*	1-M A I V V V **G** A **G** T A **G** V N A **A** F W
D-Amino acid Ox	Porcine	1-M R V V V I **G** A **G** V I **G** L S T **A** L C
Adrenodoxin R	Bovine	1-S T Q E Q T P Q I C V V **G** S **G** P A **G** F Y T **A** Q H
Fingerprint of ADP-binding site		• • • • **G** **G** • **G** • **A**

Fig. 6. The N-terminal sequence of NADH-quinone reductase. The N-terminal sequence of 25 amino acid residues of NADH-quinone reductase is aligned with those of other flavoproteins and the fingerprint of ADP-binding domain is indicated by the boldface. In the consensus sequence, (*) indicates a hydrophobic residue. The abbreviations used are as follows: DHase, dehydrogenase; Ox, oxidase; R, reductase. The sequences presented are those of dihydrolipoamide dehydrogenase from *Clostridium sporogenes* [32], sulfide-quinone reductase from *Oscillatoria limnetica* [29], glutathione reductase from *Escherichia coli* [8], NADH dehydrogenase from *E. coli* [33], rubredoxin reductase from *Pseudomonas oleovorans* [34], porcine D-amino acid oxidase [35], and bovine adrenodoxin reductase [36].

contained FMN, therefore, the conserved region was likely to be determined to occur for the binding of ADP-moiety of NAD(H). It was suggested that tryptophan residue is for FMN-binding site from the results of the photo-oxidation study [28]. On the other hand, it was reported that FMN-binding regions of several flavodoxins contained conserved tyrosine residue which were bound to the alloxazine ring of FMN [29]. Interestingly, of quinone reductases, the NADH-quinone reductase from *Streptomyces* and sulfide-quinone reductase from *O. limnetica* [30] contained this conserved region, while NAD(P)H-quinone reductase from rat liver did not contain this sequence [31].

Acknowledgement

This work was supported by the research grant 1994 of Ministry of Education, Republic of Korea.

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