Roles of Bound Quinone in the Single Subunit NADH-Quinone Oxidoreductase (Ndi1) from Saccharomyces cerevisiae*

To understand the biochemical basis for the function of the rotenone-insensitive internal NADH-quinone (Q) oxidoreductase (Ndi1), we have overexpressed mature Ndi1 in Escherichia coli membranes. The Ndi1 purified from the membranes contained one FAD and showed enzymatic activities comparable with the original Ndi1 isolated from Saccharomyces cerevisiae. When extracted with Triton X-100, the isolated Ndi1 did not contain Q. The Q-bound form was easily reconstituted by incubation of the Q-free Ndi1 enzyme with ubiquinone-6. We compared the properties of Q-bound Ndi1 enzyme with those of Q-free Ndi1 enzyme, with higher activity found in the Q-bound enzyme. Although both are inhibited by low concentrations of AC0–11 (IC₅₀ = 0.2 μM), the inhibitory mode of AC0–11 on Q-bound Ndi1 was distinct from that of Q-free Ndi1. The bound Q was slowly released from Ndi1 by treatment with NADH or dithionite under anaerobic conditions. This release of Q was prevented when Ndi1 was kept in the reduced state by NADH. When Ndi1 was incorporated into bovine heart submitochondrial particles, the Q-bound form, but not the Q-free form, established the NADH-linked respiratory activity, which was insensitive to piericidin A but inhibited by KCN. Furthermore, Ndi1 produces H₂O₂ as isolated regardless of the presence of bound Q, and this H₂O₂ was eliminated when the Q-bound Ndi1, but not the Q-free Ndi1, was incorporated into submitochondrial particles. The data suggest that Ndi1 bears at least two distinct Q sites: one for bound Q and the other for catalytic Q.

Alternative NADH dehydrogenases (NDH-2)³ catalyze electron transfer from NADH to quinone (Q) without the energy transduction. NDH-2 is considered to be composed of a single polypeptide, houses FAD as a cofactor (1, 2) and is regarded to have the simplest structure among the NADH dehydrogenases (2). The NDH-2 enzymes are present in bacteria, plant, and fungal mitochondria but not in mammalian mitochondria. In bacteria, all NDH-2 enzymes known to date are located in the cytoplasmic phase. By contrast, plant and fungal mitochondria possess two types of NDH-2 enzymes (3); one is directed to the matrix and catalyzes NADH oxidation in the matrix (designated the internal NADH dehydrogenase or Ndi), whereas the other faces the intermembrane space and oxidizes NADH in the cytoplasmic space (designated the external NADH dehydrogenases or Nde). The Ndi1 enzyme is similar to complex I in terms of NADH oxidation in the matrix (4).

A series of studies in our laboratory suggest that the Saccharomyces cerevisiae NDI1 gene may work as a therapeutic agent for mitochondrial diseases caused by complex I deficiencies (5–13). In fact, the Ndi1 expression in the substantia nigra of mouse brains has protective effects against Parkinsonian symptoms caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment (12, 14). Recently, we illustrated that the expressed Ndi1 enzyme may play a dual role in rescuing complex I-deficient cells (15); one is to restore the NADH oxidase activity, and the other is to decrease oxidative damage by complex I inhibition. It is important to clarify the mechanism of protective effects by the expressed Ndi1 against complex I deficiency. For this purpose, thorough biochemical studies of the isolated Ndi1 enzyme is a prerequisite. Although NDH-2 from several sources have been investigated, these studies provided only fragmental information about the structure and function of NDH-2 (1–3, 16–22). Therefore, overexpression and purification of the native Ndi1 enzyme seems to be indispensable.

In our earlier work (23), we overexpressed the T7 tag-fused mature Ndi1 enzyme in Escherichia coli membranes. Although we showed that the expressed Ndi1 works as a member of the respiratory chain of E. coli, the attempt to isolate the T7 tag-fused mature Ndi1 enzyme in an active form was unsuccessful. In this paper, we report that we have successfully overexpressed Ndi1 in E. coli membranes using the N-terminal His₁₀ tag fusion system and purified the functional enzyme. The purified recombinant Ndi1 has similar properties to the original Ndi1.

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3 The abbreviations used are: NDH-2, alternative NADH-quinone oxidoreductase; Ndi1, internal rotenone-insensitive NADH-quinone oxidoreductase from S. cerevisiae; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; DM, dodecyl-β-o-maltoside; Q, quinone; UQ, ubiquinone; MQ, menaquinone; SMP, submitochondrial particles; Ni-NTA, nickel-nitriotriacetic acid; Mops, 4-morpholinepropanesulfonic acid; HPLC, high pressure liquid chromatography.
enzyme isolated from *S. cerevisiae* mitochondria (24). When Triton X-100 was used for extraction of membranes, the purified Ndi1 contained no bound Q. The reconstitution of the Q-bound Ndi1 from the Q-free Ndi1 with Q was successful. From comparison studies between the Q-bound and Q-free Ndi1 enzymes, we demonstrate that the bound Q site is distinct from the Q catalytic site. Furthermore, possible roles of the bound Q in the Ndi1 are discussed.

**EXPERIMENTAL PROCEDURES**

*Materials—* UQ$_{10}$, UQ$_{2}$, UQ$_{6}$, and UQ$_{1}$ were purchased from Sigma. AC0–11 was synthesized as described by Miyoshi *et al.* (25). Dodecyl-β-d-maltoside (DM) was from Anatrace. Materials for PCR product purification, gel extraction, and plasmid preparation were from Qiagen. All of the chemicals were reagent grade and obtained from commercial sources. Bovine heart mitochondria were kindly provided by Prof. Chang-An Yu (Oklahoma State University, Stillwater, OK), and bovine heart SMP were prepared according to Ref. 26. Piericidin A$_1$ was a generous gift from Prof. Dale L. Boger (The Scripps Research Institute) (27).

**Molecular Cloning of the NDI1 Gene and Construction of Expression Plasmid—** To obtain the NDI1 gene coding for the mature form of the protein, we designed two oligonucleotide primers. One was to generate a NdeI recognition site at the mature protein initiation codon: 5'-TAGTCAGATGC-GTATCCATATGGTCCAGACAGGGTTGG-3' (the underlined bases were altered from *S. cerevisiae* DNA, and italic bases indicate the NdeI site). The other was to generate a XhoI recognition site downstream of the mature protein terminal codon: 5'-TTCAGAAGGGCATGCTCAGATGTCAATCTATATCC-3' (the underlined bases were altered from *S. cerevisiae* DNA, and italic bases indicate the XhoI site). The NDI1 gene was amplified by PCR using pRVS2.3, which has a SalI/SmaI fragment containing the full-length NDI1 gene (23) and the two primers described above. The PCR product of the NDI1 gene was subcloned into pPCR-Script Amp SK(+) plasmid and was verified by DNA sequencing. The resulting plasmid was digested with NdeI/XhoI. The NdeI/XhoI DNA fragment was finally ligated into the NdeI/XhoI site of pET16b, which is designed for expression of the N-terminal His$_{10}$ tag-fused protein. The resulting plasmid was designated pET16b(NDI1-m).

**Expression and Purification of Ndi1—** *E. coli* strain BL21 (DE3)pLyS5 was transformed with pET16b(NDI1-m). The optimum expression procedure of the His$_{10}$ tag-fused mature Ndi1 enzyme was as follows. The cells were grown in 5 ml of 2× YT medium containing 100 μg/ml ampicillin for about 7–8 h with rotation at 250 rpm at 37 °C and were used to inoculate 500 ml of the same medium. The cells were grown at 250 rpm at 37 °C until A$_{600}$ reached 0.8, and then 1 mM isopropyl β-d-thiogalacto-ryanoside was added to the culture. The cells were cultured at 70–80 rpm at 35 °C for 10 h, collected by centrifugation at 5,400 × g for 10 min, and suspended at 10 mg/ml in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The cells were then broken by sonication for 3 min at 50% duty cycle and two passages through a French pressure cell (Spectronic Instruments, Rochester, NY) at 16,000 p.s.i. Unbroken cells and inclusion bodies were removed by centrifugation at 4,300 × g for 10 min, and the supernatant was centrifuged at 250,000 × g for 60 min. The pellet (membrane fraction) was suspended at 5 mg of protein/ml in 50 mM Tris-HCl (pH 8.0 at 4 °C), 200 mM NaCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol (buffer A). After the addition of Triton X-100 at a final concentration of 0.3% (w/v), the sample was incubated for 30 min with slow stirring and centrifuged at 250,000 g for 60 min. The supernatant was applied onto a Ni-NTA column (about 1 ml of bed volume/25 mg of protein; Qiagen) and equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with 10 column volumes of buffer A containing 0.1% Triton X-100 and 15 mM histidine, and the enzyme was eluted with buffer A containing 0.02% Triton X-100 and 175 mM histidine. The active fraction was pooled and concentrated (Amicon-Ultra, molecular weight cut-off 30K; Millipore) to >10 mg of protein/ml. The concentrated Ndi1 enzyme was immediately applied onto a desalting column (Econo-Pac 10DG, 10 ml; Bio-Rad), which had been equilibrated with 50 mM Mops-KOH (pH 7.0) containing 0.1 mM EDTA, 10% glycerol, and 0.02% Triton X-100, to prevent precipitation caused by high concentration of histidine. The desalted enzyme fraction was quickly frozen in liquid nitrogen and stored at −80 °C until use.

The Ndi1 enzyme devoid of Triton X-100 was prepared by two different methods. The first method was to replace Triton X-100 with DM in the above procedures. In this case, the yield of Ndi1 decreased severalfold because DM-extracted Ndi1 did not bind to the Ni-NTA column efficiently. To improve the low yield by the first method, the second method was introduced as follows. Triton X-100 was used until the step of loading Ndi1 on the Ni-NTA column as described above. In all the subsequent procedures including the washing and elution of the Ni-NTA column, DM in buffer A was used throughout. The yield of Ndi1 thus prepared was about the same as that obtained with Triton X-100. The Triton X-100-free sample was used for measurements of UV-visible absorption spectra.

**Enzyme Assays—** NADH-UQ$_{10}$ oxidoreductase activity was measured spectrophotometrically by monitoring oxidation of NADH at 340 nm (ε = 6.2 mM$^{-1}$ cm$^{-1}$) at 30 °C. The reaction mixture (total, 0.5 ml) consisted of 50 mM sodium phosphate (pH 6.0), 1 mM EDTA, 100 μM NADH, 60 μM UQ$_{1}$, and enzyme. The reaction was started by the addition of NADH or UQ$_{1}$. Prior to the reaction, the enzyme was preincubated with the other substrate (UQ$_{1}$ or NADH) for 1 min. For the NADH oxidase activity, UQ$_{1}$ was omitted.

**Quantitation of Bound Quinones in the Purified Ndi1—** The bound Q in the purified Ndi1 enzyme were determined according to Elias *et al.* (28). Briefly, a suspension containing ~10 nmol of enzyme and 10 nmol UQ$_{10}$ as an internal standard in 100 mM NaCl was treated with 10 volumes of ethanol and incubated for 1 h at 30 °C. The denatured protein was removed by centrifugation. Two and a half volumes of n-hexane was added to the supernatant, and the suspension was vigorously mixed for 2 min by vortex. The upper phase was collected and dried under argon gas. The residue was dissolved in ethanol/methanol/acetonitrile (4:3:3 v/v/v) and applied to the reverse-phase HPLC column (Waters Spherisorb ODS2, 5 μ, 4.6 × 150 mm; Millipore) at a flow rate of 1 ml/min at 25 °C. The elution was...
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monitored at 214 nm, and the content (UQ₆) was calculated from the peak area by comparison with authentic UQ₆. The efficiency of extraction was also verified by the internal standard UQ₆ in each measurement.

Incorporation of UQ₆ into the Purified Ndi1—The purified Ndi1 (30 μm) was mixed with 60 μm UQ₆ in 50 mM Mops-KOH (pH 7.0) buffer containing 0.1 mM EDTA and 10% glycerol for 5 min, and then 0.1% DM was added to the mixture to reduce nonspecific Q binding by Triton X-100 contained in the sample. The mixture was incubated for 10–12 h at 4 °C. After centrifugation, the supernatant was applied to the desalting column (Econo-Pac 10DG, 10 ml) equilibrated with the same buffer containing 0.02% DM. The eluted fraction was concentrated (Amicon-Ultra, molecular weight cut-off 30K; Millipore) to about 10 mg of protein/ml and quickly frozen in liquid nitrogen and stored at −80 °C until use.

Reconstitution of NADH Oxidase Activity by Ndi1 and Bovine Heart SMP—First, SMP were suspended in an assay medium consisting of 50 mM sodium phosphate (pH 7.5) and 1 mM MgCl₂. Then the Ndi1 enzyme was added to the mixture. In less than 10 s after addition of the Ndi1 enzyme, the NADH oxidase activity measurement was started by NADH (100 μm). Short incubation of Ndi1 with SMP was required to exclude the possibility that the Q-free Ndi1 enzyme is associated with the bountiful Q in SMP and transformed to the Q-bound Ndi1 state. In the experiments in which inhibition of electron transfer of SMP is required, pipericidin A₁ (20 nm) or KCN (2 mm) was added before the Ndi1 addition. All of the procedures were performed at 30 °C.

Detection of Superoxide and Hydrogen Peroxide—Production of superoxide radicals induced by NADH oxidase activity of Ndi1 was determined by the superoxide dismutase-sensitive acetylated cytochrome c reduction (29). The reaction was performed in 0.5 ml of 50 mM sodium phosphate (pH 7.5) buffer, 1 mM MgCl₂, 100 μM NADH, and 20 μM acetylated cytochrome c from horse heart (Sigma) in the presence or absence of 300 units/ml superoxide dismutase (from bovine erythrocytes; Sigma). Acetylated cytochrome c reduction was monitored spectrophotometrically at 550–540 nm (ε = 19.1 M⁻¹ cm⁻¹) at 30 °C.

The production of hydrogen peroxide coupled to NADH oxidase of the Ndi1 in the absence or presence of SMP was measured using a horseradish peroxidase-based scopoletin assay, which was modified from the horseradish peroxidase-based o-dianisidine assay (30). The initial reaction was performed in the assay buffer for NADH-UQ₆ oxidoreductase and NADH oxidase activity (see “Enzyme Assays”). After NADH was completely oxidized, an appropriate volume of sample (50–200 μl) from the initial solution was transferred to a new cuvette filled with the same buffer containing 7.5 units/ml horseradish peroxidase (type VI, sigma) and 5 mm scopoletin to a final volume of 1 ml. The fluorescence change of scopoletin (excitation, 365 nm; emission, 450 nm) was measured in a SpectraMax fluorescence spectrophotometer (Molecular Devices) (31). The concentration of hydrogen peroxide of the initial solution was determined from a standard curve (0.25–5 μM hydrogen peroxide, R² = 0.999).

FIGURE 1. UV-visible absorption spectrum of the purified Ndi1. An Ndi1 preparation free of Triton X-100 (5 μm) was analyzed at 25 °C in a buffer containing 50 mM Mops-KOH (pH 7.0), 0.1 mM EDTA, 10% (w/v) glycerol, and 0.02% DM. Inset, the purified Ndi1 (5 μg) on the 11% SDS gel electrophoresis visualized with Coomassie Brilliant Blue staining.

RESULTS

Expression and Purification of Ndi1—When E. coli cells containing pET16b(NDI1-m) were grown aerobically (at 250 rpm), the expression level of the Ndi1 protein was detectable but significantly low. Lowering the rotation speed of the culture from 250 rpm to 70–80 rpm, after isopropyl β-D-thiogalactopyranoside induction, increased the expression level of Ndi1 5–10-fold. NADH oxidase activity of E. coli membranes was also increased several times, suggesting that the Ndi1 enzyme expressed in the E. coli membranes is functionally active. The inactivation during the purification process was completely protected by 10% glycerol. By using histidine instead of imidazole for the Ndi1 elution from the Ni-NTA column, we were able to prevent precipitation of the enzyme. Similar strategies were reported for purification of the His-tagged bc₁ complex (36). Approximately 10 mg of Ndi1 was yielded from 20 g of wet cells. The isolated Ndi1 protein exhibited a single band in the SDS gel (Fig. 1, inset).

Molecular Properties of Purified Ndi1—The purified Ndi1 exhibited a sharp peak at 274 nm and two broad peaks at 383 and 448 nm (Fig. 1). The apparent molecular extinction coefficients...
cents of peaks at 383 and 448 nm were 9.85 and 9.26 (mM cm$^{-1}$), respectively. The visible absorption spectra of the expressed Ndi1 are consistent with those of Ndi1 isolated from *S. cerevisiae* mitochondria (24). The peak at 448 nm was quenched by dithionite similar to authentic Ndi1 (24).

The apparent molecular mass of the expressed Ndi1 was determined to be 54 kDa by using the molecular exclusion column. This is close to the predicted molecular weight of 56,904, indicating that the expressed Ndi1 enzyme exists as a monomer. FAD content of the purified Ndi1 assessed by the heating method was 0.993 ± 0.019 mol/mol of protein. These results were confirmed by the trichloroacetic acid extraction method with SDS. The expressed Ndi1 enzyme bears one noncovalently bound FAD as a cofactor.

It is known that UQ$_8$ and MQ$_8$ are major Q components in *E. coli* (37). We examined whether the purified Ndi1 contains Q and which Q if it does. The samples prepared by Triton X-100 extraction did not contain Q. In contrast, the Ndi1 extracted by DM exhibited detectable UQ and MQ peaks on HPLC with the same retention time as those of UQ$_8$ and MQ$_8$ extracted from the *E. coli* membranes. The UQ$_8$ content of this preparation was estimated to be 0.17 mol of UQ$_8$/mol of Ndi1. The MQ$_8$ content could not be determined because of a lack of MQ standards. Because the isolated Ndi1 enzyme showed NADH-MQ reductase activity (24), it was not surprising that MQ$_8$ was associated with the expressed Ndi1 similar to UQ$_8$. The observation that the Ndi1 houses bound Q is consistent with the previous reports for *E. coli* NDH-2 extracted with cholate (17). In addition, there are known Q-bound enzymes, in which Triton X-100 extraction removed bound Q (28, 38).

**Incorporation of UQ$_6$ into the Purified Ndi1**—Because the Ndi1 enzyme extracted by DM contained a substoichiometric amount of UQ$_8$ and MQ$_8$, we tried the incorporation of Q into purified Ndi1 extracted with Triton X-100 (Q-free Ndi1). We used UQ$_6$, an authentic Q, in yeast mitochondria. As shown in Fig. 2, when the concentration of UQ$_6$ was 60 μM, the ratio of bound UQ$_6$/Ndi1 reached ~1.1. This ratio was slightly increased at higher concentrations of UQ$_6$. The slight increase of bound UQ$_6$ appears to be due to nonspecific binding of UQ$_6$ to detergent micelles that are present in the Ndi1 preparation. It should be noted that the experiments have been done with a high concentration (30 μM) of the Ndi1 enzyme. The results suggest that Ndi1 bears at least one Q-binding site.

The Q-bound Ndi1 (1 mol/mol) prepared from the Q-free Ndi1 and UQ$_6$ was subjected to UV-visible absorption spectrum analyses. As shown in curve c in Fig. 3, the difference spectrum between the Q-bound and Q-free Ndi1 clearly displayed a UQ peak with small splits (273, 283, and 290 nm). Although these splits in UV-visible absorption spectrum of the bound Q have never been reported as far as our present knowledge is concerned, it seems likely to be caused by the interaction between the bound UQ$_6$ and Ndi1.

**Kinetic Analyses of the Q-bound and Q-free Ndi1**—Our preparation extracted with Triton X-100 (Q-free Ndi1) has 2000 μmol of NADH oxidized/min/mg for NADH-UQ$_2$ reductase activity, which is comparable with 1671 μmol of NADH oxidized/min/mg reported for the Ndi1 extracted with Triton X-100 from the yeast mitochondria under the same assay conditions (24). The optimum pH of the enzyme activities of the expressed Ndi1 was found to be ~6.5, which was similar to 6.2
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FIGURE 4. Effects of preincubation with NADH on the NADH-UQ1 reductase activity of the Q-free and Q-bound Ndi1 enzymes. NADH, Ndi1 was preincubated with 60 μM UQ₁ for 1 min, and assays were started by addition of 100 μM NADH; Ndi1, the assays were started by addition of the enzymes; Q(5 s)–Q(90 s), Ndi1 was preincubated with 100 μM NADH for 5, 10, 15, 30, 60, and 90 s, respectively, and then the assays were started by the addition of 60 μM UQ₁, 100% NADH-UQ₁ reductase activities were 1100±50 mol of NADH oxidized/min/mg of Ndi1. The assays were performed at 30 °C. The values represent the averages of three measurements.

of the Ndi1 isolated from yeast mitochondria. It is likely that our overexpressed Ndi1 enzyme in E. coli displayed similar characteristics to the authentic enzyme isolated from yeast mitochondria.

To characterize the difference between the Q-bound and Q-free Ndi1, we measured the NADH-UQ₁ reductase activity. For detailed analyses of kinetic parameters in this study, we used UQ₁ as an electron acceptor instead of UQ₂ and UQ₆ for the following reasons. First, it is difficult to accurately measure NADH-UQ₂ and NADH-UQ₆ reductase activities because the Q-free Ndi1 activity. Preincubation with NADH had little or no effect on the activity of the Q-free Ndi1. Overall the Q-bound Ndi1 showed the NADH-UQ₁ activity ~30% higher than the Q-free Ndi1. These results suggest that incubation of Q-bound Ndi1 with NADH may cause release of the bound Q from Ndi1 and that the Q-free Ndi1 could be converted to the Q-bound form by recruiting Q from the reaction mixture during preincubation with Q. Therefore, we tentatively assume that, in kinetic analyses, the NADH start assay represents the characteristics of the Q-bound Ndi1, whereas the UQ₁ start assay resulted from the nature of Q-free Ndi1.

The apparent Kₚ values for NADH in NADH-UQ₁ reductase activities of the purified Q-free Ndi1 enzyme were 12 and 10 μM in the NADH and UQ₁ start assay, respectively (data not shown). These values are similar to 9.4 μM in NADH-2,6-dichlorophenolindophenol reductase activity (39) but distinct from 31 μM in NADH-UQ₁ reductase activity (24). The apparent Kₚ values for UQ₁ in NADH-UQ₁ reductase activity are 21 and 26 μM in the NADH and UQ₁ start assay, respectively (Fig. 5). As far as apparent Kₚ values are concerned, the Q-bound Ndi1 is akin to the Q-free Ndi1.

Lineweaver-Burk plots and Hanes-Woolf plots of NADH-UQ₁ reductase activity of the purified Q-free Ndi1 in the NADH start assay showed a typical ping-pong reaction (Fig. 5, A and B), as reported for the NADH-2,6-dichlorophenolindophenol reductase activity of the yeast Ndi1 enzyme (39). A similar mechanism has been postulated for NDH-2 of Mycobacterium tuberculosis (18) and the external NDH-2 of Yarrowia lipolytica mitochondria (22), suggesting that the ping-pong reaction mechanism might be common in NDH-2. In contrast, in the UQ₁ start assay the kinetic patterns of NADH-UQ₁ reductase activity were not simple and showed considerable substrate inhibition at lower concentrations of NADH (Fig. 5, C and D). At 100 μM of NADH, the substrate inhibition was barely observable.

The isolated Ndi1 also exhibited a low NADH oxidase activity. The maximum activity of NADH oxidation was 5.5 μmol of NADH oxidized/min/mg at the optimum pH of 4.5. This activity was 0.0000000000000000000000047 times of the total NADH-UQ₁ reductase activity (24). Therefore, we investigated inhibitory effects of quinolone derivatives on NADH-UQ₁ reductase activity of the purified Ndi1 (Fig. 6A). The IC₅₀ values for flavone, HQNO, and ACO–11 were ~40, 9, and 0.2 μM, respectively, regardless of whether the inhibitory assay was performed in the NADH start or UQ₁ start assay. Because ACO–11 showed a strong inhibitory effect against the Ndi1 enzyme, we studied its inhibitory mode. In the NADH start assay, ACO–11 showed a mixed mode of competitive and noncompetitive inhibition (Kᵢ = 220 nM; αKᵢ = 150 nM) (Fig. 6B), whereas ACO–11 behaved as a non-competitive inhibitor in the UQ₁ start assay.
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![Image](https://example.com/image.png)

**Figure 5.** Kinetics analysis of the NADH-UQ oxidoreductase activity of Ndi1. NADH-UQ oxidoreductase activities were measured with varied concentrations of UQ and were analyzed in Lineweaver-Burk plots (A and C) and Hanes-Woolf plots (B and D). The reaction was started by the addition of NADH (A and B) or UQ (C and D). Panels A and B, NADH concentration was 10 μM (△), 15 μM (▲), 20 μM (○), and 100 μM (●). Panels C and D, NADH concentration was 5 μM (△), 10 μM (▲), 15 μM (○), and 100 μM (●).

From the results presented in Fig. 4, we postulated that incubation of the Q-bound Ndi1 with NADH may cause dissociation of Q from the Ndi1 enzyme. To verify this possibility, we examined the amount of bound UQ6 in Ndi1 after incubation with NADH. Because of the intrinsic NADH oxidase activity of Ndi1, the experiments were carried out under strict anaerobic conditions. The addition of NADH or dithionite to the Q-bound Ndi1 released ~75% of the bound UQ6 from Ndi1 (Fig. 7); similar effects were seen with NADPH (data not shown). The addition of NAD had no effect on the bound UQ6. We noticed the appearance of a yellow color of Ndi1 during the desalting step that separates the dissociated UQ6 from Ndi1, indicating the transition of the redox state of Ndi1 (FAD). When we included NADH in the desalting step to keep the Ndi1 in the reduced form, the dissociation of bound UQ6 was prevented (Fig. 7). In contrast, adding NAD to the desalting step had no effect on the dissociation of bound UQ6 from Ndi1 by NADH. Therefore, it is strongly suggested that the bound UQ6 tightly associates with Ndi1 in the oxidized state but not during the redox transition. However, as long as the substrate site is occupied with NADH and FAD remains reduced, the bound UQ6 is retained in the Ndi1 enzyme.

**Introduction of Ndi1 Enzyme into the Respiratory Chain of Bovine Heart Submitochondrial Particles—**To gain insight into the physiological role of the bound Q, we performed a series of reconstitution experiments using bovine heart SMP. The preparation of bovine SMP used for the reconstitution had an NADH oxidase activity of 1.2 μmol of NADH oxidized/min/mg of SMP. The NADH oxidase activity of SMP was completely inhibited by 20 nM piericidin A1 or 2 mM KCN. To exclude the contribution from endogenous complex I, we measured the NADH oxidase activity in the presence of piericidin A1. As shown in Fig. 8A, both the Q-free and the Q-bound Ndi1 enzyme in the absence of SMP exhibited NADH oxidase activity of ~2.6 μmol of NADH oxidized/min/mg of Ndi1 (lanes 1 and 2).

When the Q-bound Ndi1 was incorporated into SMP, the reconstituted preparation exhibited the NADH oxidase activity of 11 μmol NADH oxidized/min/mg of Ndi1 (lane 3). This activity was inhibited by KCN, indicating that the oxidation of NADH by Ndi1 is now connected to the respiratory chain of the mitochondrial membrane. It should be noted that antimycin A also inhibited this activity (data not shown). To ascertain this point, we measured production of H2O2 associated with NADH oxidase by Ndi1 (Fig. 8B). Both the Q-free and the Q-bound enzymes in the absence of SMP produced H2O2 that accounts for 96% of NADH added to the assay (lanes 1 and 2). In addition, the rate of superoxide production by the Q-bound and the Q-free Ndi1 was determined to be 0.28 μmol of superoxide radicals/min/mg of Ndi1, which accounted for only ~5% of the NADH oxidase activity. The data indicate that H2O2 is the main product of the NADH oxidation by Ndi1 when the electron acceptor is oxygen. This H2O2 production was totally suppressed when the Q-bound Ndi1 was bound to SMP (* in lane 3 of Fig. 8B). Apparently, under these conditions, oxygen is no longer a substrate for Ndi1, and the electrons are donated to the downstream respiratory chain of SMP. In fact, when the respi-

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4 If the Q-free Ndi1 is incubated with SMP for a few minutes, it tends to show the characteristics of the Q-bound Ndi1, presumably by incorporating UQ6 present in SMP.
The His-tagged Ndi1 enzyme expressed in and purified from E. coli membranes exhibited characteristics similar to those of the authentic Ndi1 isolated from yeast mitochondria in terms of enzymatic activities, kinetic parameters, and cofactor contents. Overexpression of the Ndi1 allowed us to prepare enough of the purified enzyme that is functional and suitable for study of its properties.

By a combination of selection of the detergent used for the isolation and incubation with externally supplied Q, we were able to obtain the Q-free form and the Q-bound form of Ndi1. The two forms of the Ndi1 were then used to investigate the nature of the Q-binding site. A question arises of whether the bound Q is at the catalytic site. This did not seem to be the case. First, addition of NADH, NADPH, or dithionite under anaerobic conditions released bound Q from Ndi1 presumably when bound Q was reduced. However, the bound Q was retained on Ndi1 as long as the substrate site is occupied with NADH that can keep FAD in the reduced state by charge transfer. Second, the apparent rate constant of the release of bound Q from Q-bound Ndi1 by NADH was very slow (~0.14 s⁻¹), whereas turnover of the enzyme reaction of Ndi1 was significantly faster (~900 s⁻¹), indicating that bound Q is not kinetically competent. Clearly, these properties of the bound Q are distinct from those of Q as the substrate, suggesting that there are two Q-binding sites (bound Q site and catalytic site) in Ndi1. However, the binding experiment seems to imply only 1 mol of Q/mol of enzyme. The most likely reason is as follows. The kinetic analyses of NADH-UQ₁ reductase activity of the Q-bound Ndi1 (Fig. 5, A and B) suggest the mechanism of a typical ping-pong reaction (Scheme 1).

In this reaction scheme, binding of the substrate Q to the catalytic site only occurs after a sequence of events that includes binding of NADH to the enzyme, reduction of the enzyme (probably FAD) by NADH, and release of the product NAD. Under the conditions employed for the binding experiment, Ndi1 (FAD) was in the oxidized form, and therefore, binding of Q to the catalytic site did not take place. In 1981, Jaworowski et al. (17) reported that two different preparations of the E. coli NDH-2 isolated with cholate contained 0.67 and 1.1 mol of UQ/mol of enzyme. Although many papers concerning NDH-2...
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FIGURE 7. Effects of NADH, NAD, and dithionite on the dissociation of UQ from Q-bound Ndi1. All of the experimental steps were carried out in an anaerobic chamber at room temperature. The Q-bound Ndi1 (30 μM in 0.5 ml) was treated with nothing, NAD⁺, NADH, or dithionite for 1 min in a buffer containing 50 mM MOPS-KOH (pH 7.0), 0.1 mM EDTA, 10% (v/v) glycerol, and 0.02% DM, and then dissociated UQ was removed by a desalting column equilibrated with the same buffer with or without NADH or NAD⁺. Eluate from the desalting column was assayed for bound UQ by reverse-phase HPLC. The 100% of bound UQ was the initial amount of bound UQ before experimental conditions. Therefore, it seems likely that Ndi1 another important consequence in light of our situ bears bound Q to facilitate electron transfer from NADH to another site.

have been published (1–3, 19, 44), only this paper was concerned with the bound UQ of the enzyme. It might be possible that the bound UQ in E. coli NDH-2 is also not on the catalytic site.

Little is known about roles of the Q-binding sites of NDH-2 (1, 2). However, the existence of two distinct Q-binding sites was reported in E. coli membrane-bound glucose dehydrogenase containing pyrroloquinoline quinone as a cofactor and composed of a single polypeptide (28). The bound Q in membrane-bound glucose dehydrogenase is considered to be involved in electron transfer from pyrroloquinoline quinone to UQ₈ pool (45). Because semiquinone in Ndi1 has not been measured yet, it is at present uncertain whether the bound Q of Ndi1 participates in the electron transfer from FAD to Q pool. However, the importance of bound Q in Ndi1 was demonstrated in the reconstitution experiment in which the presence of bound Q on Ndi1 was shown to restore the KCN- and antimycin A-sensitive NADH oxidase activity of SMP. In addition, NADH oxidase of SMP reconstituted with the Q-bound Ndi1 did not produce H₂O₂, indicating that the added Q-bound Ndi1 was all coupled to the respiratory chain of SMP under our experimental conditions. Therefore, it seems likely that Ndi1 in situ bears bound Q to facilitate electron transfer from NADH to Q pool.

The reconstitution experiment using bovine SMP has another important consequence in light of our in vivo studies using the Ndi1. We have demonstrated that the Ndi1 can be introduced into mammalian mitochondria as an alternative NADH dehydrogenase for the respiratory chain and is able to complement defective complex I both in cultured cells and in animals (5, 8, 12, 14). Successful incorporation of the Ndi1 with the bovine inner mitochondrial membrane in vitro should provide us a powerful model in which to investigate...
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detailed mechanism of association of this yeast enzyme and the mammalian mitochondria under designated and closely controlled conditions. In this regard, it is apparent that our preparation of the Ndi1 is suitable for testing its functionality and will provide a solid basis for studies at the cellular and tissue level.

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