Electron Transfer Ability from NADH to Menaquinone and from NADPH to Oxygen of Type II NADH Dehydrogenase of *Corynebacterium glutamicum*

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Type II NADH dehydrogenase of *Corynebacterium glutamicum* (NDH-2) was purified from an *ndh* overexpressing strain. Purification conferred 6-fold higher specific activity of NADH:ubiquinone-1 oxidoreductase with a 3.5-fold higher recovery than that previously reported (K. Matsushita et al., 2000). UV–visible and fluorescence analyses of the purified enzyme showed that NDH-2 of *C. glutamicum* contained non-covalently bound FAD but not covalently bound FMN. This enzyme had an ability to catalyze electron transfer from NADH and NADPH to oxygen as well as various artificial quinone analogs at neutral and acidic pHs respectively. The reduction of native quinone of *C. glutamicum*, menaquinone-2, with this enzyme was observed only with NADH, whereas electron transfer to oxygen was observed more intensively with NADPH. This study provides evidence that *C. glutamicum* NDH-2 is a source of the reactive oxygen species, superoxide and hydrogen peroxide, concomitant with NADH and NADPH oxidation, but especially with NADPH oxidation. Together with this unique character of NADPH oxidation, phylogenetic analysis of NDH-2 from various organisms suggests that NDH-2 of *C. glutamicum* is more closely related to yeast or fungal enzymes than to other prokaryotic enzymes.

Key words: type II NADH dehydrogenase; NAD(P)H–quinone oxidoreductase; superoxide; bacterial respiratory chain; *Corynebacterium glutamicum*

NADH:quinone oxidoreductase, found in bacterial respiratory chains, can be divided into three different types: a proton-translocating type I NADH dehydrogenase (NDH-1), type II NADH dehydrogenase lacking an energy coupling site (NDH-2), and Na\(^+\)-translocating NADH:quinone oxidoreductase. NDH-1, which is homologous to mitochondrial complex I, is composed of 13–14 different subunits and has FMN and several iron–sulfur clusters as the prosthetic groups. This enzyme is able to pump protons from the cytosolic side to the periplasmic side. NDH-2 is a single subunit enzyme and bears flavin but no iron–sulfur clusters.\(^\text{1)}\) Although the oxidation of NADH is extensively carried out by complex I in mammals, mitochondria from fungi contain an alternative NADH dehydrogenase, NDH-2, together with complex I in *Neurospora crassa*\(^\text{2)}\) or without complex I in *Saccharomyces cerevisiae*.\(^\text{3)}\) Similarly to fungi, the bacterial respiratory chain has NDH-1 and NDH-2, or either one of these. *Escherichia coli* has NDH-1 and NDH-2,\(^\text{4)}\) which are encoded by the *ndo* operon\(^\text{5)}\) and the *ndh* gene\(^\text{6)}\) respectively, but *Paracoccus denitrificans* has only NDH-1\(^\text{7)}\) and *Bacillus subtilis* has only NDH-2.\(^\text{8)}\) Although bacterial NDH-1 from *E. coli*,\(^\text{9,10)}\) and *P. denitrificans*,\(^\text{11,12)}\) has been well characterized, NDH-2 has not been well studied, except for *E. coli* NDH-2.\(^\text{13,14)}\) Prokaryotic NDH-2 has been isolated from *B. subtilis*,\(^\text{15)}\) *Methylococcus capsulatus*,\(^\text{16)}\) *Acidianus ambivalens*,\(^\text{17)}\) and *Sulfolobus metallicus*.\(^\text{18)}\) These enzymes lack iron–sulfur clusters and contain a flavin, non-covalently bound FAD in most cases\(^\text{13,15,16)}\) but covalently bound FMN in other cases.\(^\text{17,18)}\) There is no evidence that NDH-2 from these microorganisms contains a metal binding motif. Only *E. coli* NDH-2 has been reported to contain a Cu(I)–thiolate ligation domain.\(^\text{19)}\)

The Gram-positive coryne-form bacterium *Corynebacterium glutamicum* is an amino acid producing strain used industrially for the production of L-lysine and L-glutamate. The respiratory chain of this bacterium consists of several different primary dehydrogenases,\(^\text{20,21)}\) such as NADH dehydrogenase, succinate dehydrogenase, L-lactate dehydrogenase, and malate: quinone oxidoreductase, and at least three terminal oxidases, CN-sensitive cytochrome *aa*\(_3^\text{,22,23)}\) CN-resistant bypass oxidase,\(^\text{20)}\) and cytochrome *bd*.\(^\text{24)}\) The NADH...
Material and Methods

Materials. Ubiquinone-1 or -2 (Q₁ or Q₂) and menaquinone-2 (MQ₂) was kindly supplied by Eizai Co., Japan, and by Professor N. Sone and Dr. J. Sakamoto of the Kyushu Institute of Technology, respectively. FAD, FMN, deamino-NAD, and horse-radish peroxidase were from Sigma. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) was from Oxis (Portland, OR, U.S.A.). All other materials were of reagent grade and were obtained from commercial sources.

Bacterial strains and growth conditions. An NDH-2 over-expressing strain derived from C. glutamicum KY9714(25) was used in this study. For cultivation, the strain was inoculated into Luria–Bertani (LB) medium supplemented with 25 μg/ml kanamycin and incubated at 30°C under 200 rpm shaking overnight. The LB-grown cells were then transferred with the 1% inoculum to a 1-liter of glucose minimum medium(20) containing 25 μg/ml kanamycin in a 3-liter Erlenmeyer flask and grown at 30°C until the late exponential phase.

Membrane preparation. The cells were harvested by centrifugation and washed twice with 20 mM potassium phosphate buffer (KPB), pH 7.5. The washed cells were resuspended in the same buffer at 2.5 ml per g wet cells in the presence of 0.5 mg/ml of lysozyme and then incubated at 30°C under 80 rpm rotation for 1 h. Then the lysozyme-treated cells were disrupted by passing them twice through a French pressure cell press at 16,000 psi, and the cell debris was removed by centrifugation at 6,000 rpm for 20 min at 4°C. The supernatant was ultracentrifuged at 40,000 rpm for 30 min at 4°C. The membrane precipitated was resuspended with 50 mM KPB, pH 6.5, containing 1 mM O,O'·-Bis<2-aminoethyl>ethyleneglycol-N,N,N',N'-tetra acetic acid (EGTA), and 4 mM MgCl₂ at a final concentration of 10 ng protein per ml. The suspension was subsequently used for purification.

Purification of NDH-2. All steps were performed at 0—4°C. The membrane suspension was incubated on ice for 30 min in the presence of 2% Triton X-100 and then ultracentrifuged at 40,000 rpm for 30 min. The supernatant was directly applied on a DEAE-Toyopearl column (1 ml of bed volume per 2–3 mg of protein applied) which had been equilibrated with 50 mM KPB, pH 6.5, containing 1 mM EGTA and 4 mM MgCl₂. The column was washed with 3 bed volumes of column buffer (50 mM KPB, pH 6.5, containing 1 mM EGTA, 4 mM MgCl₂, 0.1% Triton X-100, and 20 μM FAD), followed by washing with 3 bed volumes of the column buffer containing 0.1 M KCl. Then the enzyme was eluted by a linear gradient consisting of 3 bed volumes each of the column buffer containing 0.1 M KCl and 0.3 M KCl. Enzyme activity appeared at 0.2 M KCl from the column. The pooled active fractions were dialyzed against 30-fold volumes of column buffer at 4°C for 12 h. The dialyze was applied on a Heparin-Sepharose column (1 ml bed volume per mg of protein) which had previously been washed with 50 mM KPB, pH 6.5, containing 1 mM EGTA and 4 mM MgCl₂. After the column was washed with 3 bed volumes of column buffer containing 0.15 M KCl, the enzyme was eluted with 3 bed volumes of column buffer containing 0.35 M KCl. The active fractions were then combined and used for further experiments.

Enzyme assays. All enzyme assays were performed at 25°C. NAD(P)H oxidase activity was spectrophotometrically measured at 340 nm by following the decrease in NAD(P)H concentration. The reaction mixture (1 ml) contained an appropriate amount of enzyme, 0.2 mM NAD(P)H, and 50 mM KPB or Na–acetate buffer. To see full enzyme activity, 20 μM FAD was also added to the reaction mixture when indicated. The amount of enzyme that oxidized 1 μmol of NAD(P)H per min was defined as 1 unit, where a millimolar extinction coefficient of 6.2 or 6.3 was used for the calculation of NADH or NADPH oxidase activity respectively. Q₁ or Q₂ reductase activity was spectrophotometrically measured by following the decrease in absorbance at 340 nm in a 1 ml reaction mixture consisting of an appropriate amount of enzyme, 0.2 mM NAD(P)H, 50 μM Q₁ or 30 μM Q₂, and 50 mM KPB or Na–acetate buffer. A unit of activity was defined as 1 μmol of NAD(P)H oxidized per min, calculated using a millimolar extinction coefficient of 6.81. Ferricyanide (K₃Fe(CN)₆; FR) reductase activity was spectrophotometrically measured by following the decrease in absorbance at 420 nm. The reaction mixture consisted of an appropriate amount of enzyme, 0.2 mM NAD(P)H, 1 mM K₃Fe(CN)₆, and 50 mM KPB. A unit of activity was defined as 2 μmol of K₃Fe(CN)₆ reduced
Detection of superoxide and hydrogen peroxide. Production of the superoxide radical was quantitatively determined by the reduction of cytochrome c. The reaction was performed in an appropriate buffer along with 0.2 mM NAD(P)H, 30 µM MQ; or 50 µM MD, and 50 mM KPB or Na–acetate buffer. A unit of activity was defined as 2 µmol of K3Fe(CN)6 reduced per min, and FR reduction activity without MQ2 or MD was subtracted to lead MQ2- or MD-dependent FR reductase activity. $K_m$ and $V_{\text{max}}$ values were determined from a Hofteev plot of several electron acceptors, oxygen, Q1, Q3, FR, MD, and MQ2, for NDH-2 in the presence of NADH or NADPH as an electron donor.

Spectroscopic analyses. To remove FAD and also exchange Triton X-100 with dodecylmaltoside in the purified enzyme, the purified enzyme (about 1.5 mg protein) was dialyzed against 50 mM KPB, pH 7.0, and then applied to a DEAE-Toyopearl column (1 ml bed volume) equilibrated with the same buffer. The column was washed with 10 ml of the same buffer containing 0.1% dodecylmaltoside, and the enzyme was eluted with the same buffer containing 0.1% dodecylmaltoside and 0.35 M KCl. UV–visible spectra of the purified enzyme (4 µM) were recorded over a wavelength range of 250 to 600 nm on a double wavelength spectrophotometer (Hitachi 557) at 25°C. Fluorescence spectra of purified NDH-2 were recorded at 25°C with a Hitachi 650-10S fluorescence spectrophotometer.

EPR spectra were recorded on a Bruker ELEXSYS E 500 spectrometer operating at 9.85 GHz at room temperature. The EPR conditions were typically a microwave power of 10 milliwatts and a modulation amplitude of 1.0 Gauss. A flat quartz cell (JEOL LC11, inner volume 60 µl) was used for the EPR measurement. The EPR sample contained 1.2 µM purified NDH-2, 0.2 mM FAD, 1 mM NAD(P)H, and 45 mM DEPMPO in 50 mM KPB, pH 6.5, or 50 mM Na–acetate buffer, pH 5.5. The sample was incubated for 15 min at room temperature. Hydroxyl radical (·OH) and superoxide radical (O2·−) can be trapped and stabilized by DEPMPO, and the adds show different characteristics of the EPR spectrum.29)

SDS–PAGE analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli30 followed by staining with Coomassie brilliant blue R250. The protein standard marker used consisted of phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.1 kDa), and lysozyme (14.4 kDa).

Protein determination. The concentration of protein was determined by a modified Lowry method,31 and bovine serum albumin was used as a standard.

Results

Purification of NDH-2

Although NDH-2 has previously been purified from C. glutamicum KY9714,25 the enzyme was unstable and recovery during purification was low, suggesting that FAD might have been lost. In this study, we attempted to purify the enzyme from an NDH-2 overexpressing strain derived from KY9714 and tried to improve its recovery by adding FAD throughout the purification steps. Purification was performed by two-step column chromatography using DEAE-Toyopearl and Heparin–Sepharose, as described in “Materials and Methods”. As summarized in Table 1, NADH:Q1 reductase activity was enriched 6.2-fold with a 15.6% yield. Enzyme purity was confirmed on SDS–PAGE, where a single polypeptide of 55-kDa corresponding to NDH-2 was observed (Fig. 1).

Spectral analysis of the purified enzyme

To avoid interference in spectral analysis, excess FAD supplemented during the purification was removed by dialysis and Triton X-100 was exchanged with dodecylmaltoside, as described in “Materials and Methods”. The purified enzyme thus prepared exhibited an

<table>
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<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (U)</th>
<th>Yield (%)</th>
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<td>Membrane</td>
<td>280</td>
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<td>12,320</td>
<td>100</td>
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<td>273</td>
<td>1,917</td>
<td>15.6</td>
</tr>
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</table>

Table 1. Summary of NDH-2 Purification
absorption spectrum with a maximum at 273 nm and a broad shoulder between 350 and 450 nm (Fig. 2). The peak around 450 nm was reduced by the addition of dithionite, indicating the presence of a typical flavin cofactor, but the flavin content estimated from the spectrum might have been underestimated because a large portion of flavin was detached during dialysis, as can be seen in the finding that NADH:Q1 reductase activity was reduced to 34% (from 273 to 93 U/mg) after the dialysis. The decreased activity during dialysis could not be recovered by further addition of FAD or FMN, although the reason is not known. Thus a flavin moiety attached to the dialyzed enzyme was examined by fluorescence analysis at various pHs.32) The flavin moiety extracted from the dialyzed enzyme by heat treatment exhibited a fluorescence spectrum with a single emission at 530 nm when excited at 452 nm (Fig. 3A and B). The emission maximum was observed at acidic pH (Fig. 3C), suggesting that NDH-2 of C. glutamicum contains a non-covalently bound FAD.

Although, since NDH-2 of E. coli has been shown to contain copper (Cu(I)), luminescence spectroscopy was performed with the purified enzyme, no bands corresponding to Cu(I) were observed (data not shown). In addition, metal analysis using induced-coupled plasma atomic emission spectrometry indicated that purified NDH-2 did not contain any copper (data not shown).

Kinetic properties of the purified enzyme
C. glutamicum NDH-2 oxidizes NADPH at acidic pH with or without Q1 in addition to oxidizing NADH with Q1 at neutral pH.24) In this study, we examined both
NADH and NADPH oxidations kinetically with various electron acceptors in more detail. NADH oxidation coupled with various artificial electron acceptors exhibited maximal activity at neutral pH between 6 and 7, whereas NADPH oxidation had an optimum pH under acidic conditions at about pH 5 (Fig. 4). Only NADH:Q₁ oxidoreductase activity had two optimum pHs. Therefore the kinetic properties of NDH-2 for NAD(P)H oxidation coupled with the reduction of several electron acceptors were further examined at their optimum pH (Table 2 and 3). As expected, the purified enzyme exhibited a higher affinity for NADH than for NADPH when oxygen or Q₁ was used as the electron acceptor, although the $K_m$ for NADH with oxygen could not be determined due to a low value of less than 2 μM (Table 2).

The highest affinity for the electron acceptor of the purified enzyme was observed with MQ₂ coupling for the oxidation of NADH, while the reduction of MQ₂ was not observed with NADPH oxidation (Table 3). MQ₂ reductase activity was measured as FR reduction activity via MQ₂, in which the final electron acceptor was required to maintain the higher activity. The MQ analog MD was reduced by the enzyme even with NADPH, although the affinity and activity were lower than those for NADH (Table 3). A higher affinity for the artificial

Fig. 4. pH-Dependent NAD(P)H Oxido-Reductase Activities with Various Artificial Electron Acceptors or Oxygen. The closed and open circles represent oxidation of NADH and NADPH respectively.

| Table 2. Kinetic Parameters of NAD(P)H for Purified NDH-2 |
|-------------|------------|------------|------------|---|---|
| Substrate   | $K_m$ (NADH) μM | $V_{max}$ (units/mg) | $K_m$ (NADPH) μM | $V_{max}$ (units/mg) |
| NAD(P)H oxidase | <2          | (2.13)      | 105         | 7.5           |
| NAD(P)H:Q₁ oxidoreductase | 11.6 (105) | 394 101     |             |               |

*Measured at pH 7.
**Measured at pH 5.
***The $V_{max}$ value for NADH:Q₁ reductase is not accurate due to the relatively low Q₁ concentration (50 μM) used for this assay.
electron acceptors, ubiquinone analog (Q₁ or Q₂) and FR, was observed for NADPH oxidation rather than for NADH. It should be noted that purified NDH-2 did not react with deamino-NADH (100 μM) as in the case of E. coli enzyme (less than 1% of NADH) and also that it could not be inhibited with 10 μM rotenone.

Antimycin A inhibition upon the oxidation of NAD(P)H by purified NDH-2

Since NDH-2 of C. glutamicum has an ability to donate electrons to menaquinone or ubiquinone, sensitivity to several quinone inhibitors was examined to get more insight into the reactivity of the enzyme towards quinone and oxygen. First, various different inhibitors were used to determine whether they inhibited NAD(P)H:quinone reductase activity. As for the result, the reduction of quinones with purified NDH-2 was shown to be inhibited only by antimycin A (data not shown). As shown in Fig. 5, NADH:MQ and NAD(P)H:Q₂ reductase activities were intensively inhibited with 25 μM antimycin A, but NAD(P)H oxidase activities were not. This result also indicates that NADPH:Q₂ reductase activity was slightly more sensitive to antimycin A than was NADH reductase.

Production of superoxide and hydrogen peroxide by purified NDH-2

The NADH oxidase system of C. glutamicum can produce superoxide anions. Moreover, type II NADH dehydrogenase (rotenone-insensitive NADH dehydrogenase) of Trypanosoma brucei and S. cerevisiae produce superoxide in the presence of NADH. Therefore, in this study, superoxide generation dependent on NADH or NADPH oxidation was examined using the purified NDH-2 of C. glutamicum. The production of superoxide was quantitatively determined by following the reduction of horse heart cytochrome c at various pH conditions and comparing this with the NAD(P)H oxidase activity of the enzyme (Table 4). Superoxide production was observed at pH 5 to 8 with NADPH, but not with NADH (data not shown).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NADH*</th>
<th>NADPH**</th>
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<tr>
<td></td>
<td>〈μM (acceptor)〉</td>
<td>〈 units/mg)〉</td>
</tr>
<tr>
<td>NAD(P)H:Q₁ oxidoreductase</td>
<td>41</td>
<td>256</td>
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<tr>
<td>NAD(P)H:Q₂ oxidoreductase</td>
<td>32</td>
<td>841</td>
</tr>
<tr>
<td>NAD(P)H:FR oxidoreductase</td>
<td>133</td>
<td>23.8</td>
</tr>
<tr>
<td>NAD(P)H:MD oxidoreductase</td>
<td>31</td>
<td>284</td>
</tr>
<tr>
<td>NAD(P)H:MQ₂ oxidoreductase</td>
<td>5.0</td>
<td>81.5</td>
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</table>

* Measured at pH 7 except for FR reductase activity, which was measured at pH 5.
** Measured at pH 5.
*** The Vₘₐₓ values of these activities are not accurate because the NADPH concentration used was not saturated.
**** ND, not detected.

Figure 5. Antimycin A Inhibition of NAD(P)H Oxidase, and NAD(P)H:Q₂ and NADH:MQ₂ Reductase Activities. Enzyme activities with NADH and with NADPH were measured in 50 mM KPB, pH 6.5, and in 50 mM Na–acetate buffer, pH 5.5, respectively, as described in “Materials and Methods”. NADH oxidase; NADH:Q₂ reductase; NAD(P)H:Q₂ reductase; NAD(P)H:MQ₂ reductase.
To confirm the production of superoxide during NAD(P)H oxidation, the formation of superoxide with NDH-2 was detected directly by EPR, in which superoxide was determined by the formation of DEPMPO-adduct under aerobic conditions. The EPR signal with hyperfine coupling constants of $a_N = 13.0$, $a_P = 11.1$, and $a_H = 50.0$ in Gauss was obtained in both the NADH and NADPH oxidations in the presence of purified NDH-2 (Fig. 6). The EPR signals were attributed to DEPMPOOOH, which derived from superoxide but not from the hydroxyl radical (*OH), and was not observed without NAD(P)H or NDH-2. A higher signal intensity was obtained with NADPH than with NADH at pHs 5.5 and 6.5, and also a higher signal was obtained at pH 5.5 than at pH 6.5 with NADPH. Together with the observations for cytochrome c reduction, we concluded that NDH-2 produces a superoxide radical concomitant with NADPH oxidation at acidic pH, and to a lesser extent with NADH oxidation.

Although, judging from the cytochrome c reduction, the highest efficiency of superoxide formation with NADPH appeared to occur at neutral pH, EPR analysis indicated that superoxide formation occurs parallel to oxidation activity. Since generated superoxide appears to be converted spontaneously into hydrogen peroxide under acidic conditions, we further examined the production of hydrogen peroxide from NADH and NADPH at the pH that induced the highest superoxide production and oxidase activity (Table 4). The results indicate that the oxidation of NADH and NADPH resulted in the generation of hydrogen peroxide, which may be produced from superoxide at least at pH 5 with NADPH, because superoxide generation was not very high in spite of being expected to be very high by EPR analysis under these conditions.

### Discussion

Recently, NDH-2 was purified from a lysozyme-sensitive strain of *C. glutamicum* and shown to exhibit NADPH oxidase activity in addition to NADH dehydrogenase activity. In this study, in order to characterize this enzyme in more detail, NDH-2 of *C. glutamicum* was purified from an over-producing strain by an improved purification procedure in which FAD was added throughout the purification process. After purification, NDH-2 was enriched 6.2-fold with 15% yield based on NADH-Q$_1$ reductase activity, of which the specific activity and recovery were enhanced by 6-fold and 3.5-fold respectively, compared with the values previously reported. Similarly to NDH-2, found in other organisms, the spectrum of the purified enzyme showed that *C. glutamicum* NDH-2 contains a flavin moiety. This is consistent with the sequence information on this enzyme, which shows that it contains an FAD.
binding motif. In this study, we also presented evidence that *C. glutamicum* NDH-2 contains non-covalently bound FAD but not covalently bound FMN.

Although *E. coli* NDH-2 contains thiolate-bound Cu(I) and is predicted to have two conserved cysteine residues, *C. glutamicum* NDH-2 does not contain Cu and has neither a heavy-metal-associated domain nor conserved cysteine residues in its polypeptide sequence (data not shown). NDH-2 of *E. coli* contains the predicted transmembrane domain at the C-terminal region, and the external alternative NADH dehydrogenase of *N. crassa* also contains a transmembrane domain at the N-terminal region. On the other hand, NDH-2 of *A. ambivalens* contains 3 possible putative amphipatic helices located around amino acid residues 15–27, 181–194 and 332–347, which are also present in *E. coli* NDH-2. Therefore the secondary structure of *C. glutamicum* NDH-2 was predicted using programs provided by the TMHMM (http://www.enzim.hu/hmmtop/) and Columbia University (http://www.cubic.bioc.columbia.edu) servers. The results indicated that there is a single putative transmembrane region in the C-terminal region (around amino acid residues 384–439), and also in similar possible amphipatic regions (residues 179–196, 329–346 and 442–459). Thus we cannot provide strong evidence to show how this enzyme is located on the membrane of *C. glutamicum*.

When the oxidation of NAD(P)H was examined with various electron acceptors, reduction of MD, Q, and FR was observed with both NADH and NADPH, whereas reduction of MQ was observed only with NADH. But undetectable NADPH–MQ reductase activity does not necessarily imply that this enzyme cannot reduce MQ with NADPH. Since the oxidation of NADPH by purified enzyme is very weak compared with NADH oxidation, it is conceivable that the oxidation of NADPH with MQ might be below the detection limit in the *in vitro* assay. The *K*ₘ and *V*ₘₐₓ for MQ reduction of *C. glutamicum* NDH-2 with NADH (*K*ₘ = 5 µM; *V*ₘₐₓ = 82 U/mg) were reasonably low and high respectively, whereas the affinity (*K*ₘ = 41 µM) for ubiquinone analogue, Q, is significantly lower than that in *E. coli* NDH-2 (*K*ₘ = 5.9 µM). Also the *K*ₘ values for Q₁, Q₂, and FR reduction due to NADH oxidation were higher than those due to NADPH oxidation, and thus these artificial electron acceptors are more favorable electron acceptors for coupling with NADPH oxidation. The results suggest that menaquinone is a more favorable electron acceptor for *C. glutamicum* NDH-2 than other quinones at least for NADH oxidation. Furthermore, since the purified enzyme exhibits reasonably higher oxidase activity when coupled with NADPH, the physiological electron acceptor coupled with NADPH oxidation of *C. glutamicum* NDH-2 is probably oxygen, although the actual affinity for oxygen was not determined in this study. Taken together with these results, it can be concluded that NDH-2 in *C. glutamicum* oxidizes NADH tightly coupled with a natural electron acceptor menaquinone within the membrane, while it may oxidize NADPH partly coupled with oxygen reduction. However, the results obtained with antimycin A inhibition provided evidence that *C. glutamicum* NDH-2 donates electrons from both NADH and NADPH to quinone in the same manner, or at the same site, yet with different efficiencies, whereas the oxygen reacting site may be different from the quinone site. The results obtained in this study also suggest that the binding of NADPH and/or NADH might cause a conformational change in the enzyme structure, which causes a change in the binding site for the electron acceptor, including oxygen, or that it might directly overlap the acceptor binding site so that NADPH binding instead of NADH changes the binding of the acceptors.

Various redox carriers of the electron transport chain are possible sources of reactive oxygen species formation. In mammalian mitochondria, the main sites for superoxide and hydrogen peroxide production are NADH:quinone reductase, complex I, and ubiquinol-cytochrome c reductase. Type II NADH dehydrogenase of *E. coli*, *T. brucei* or *S. cerevisiae* is also a source of endogenous superoxide production. Our study also indicates that NDH-2 of *C. glutamicum* is a potential source of superoxide and hydrogen peroxide formation. The oxidation of NADH and NADPH with oxygen, especially NADPH oxidation, with NDH-2 led to the formation of superoxide and hydrogen peroxide. Since, unlike *E. coli* enzyme, NDH-2 of *C. glutamicum* has the ability to oxidize NADPH in addition to NADH, it is possible that superoxide was generated from NADPH oxidation with NDH-2. *E. coli* enzyme can also generate superoxide from NADPH, although not as high an amount as observed for that from *C. glutamicum* (unpublished results). In contrast, a similar amount of superoxide formation from NADH using NDH-2 between *C. glutamicum* and *E. coli* detected by EPR was observed in our laboratory (unpublished). However, similarly to the *C. glutamicum* enzyme, NDH-2 of *T. brucei* and *S. cerevisiae* produce a relatively high superoxide from NAD(P)H and NADH respectively.

NDH-2 of *C. glutamicum* exhibits a unique characteristic, NADPH oxidation, that occurs more often in eukaryotic enzymes than in prokaryotic ones. The external alternative NADH dehydrogenases NDE-1 and NDE-2 of *N. crassa* oxidize NADPH at acidic pH, similarly to the *C. glutamicum* enzyme. Therefore, a phylogenetic tree of NDH-2 from various organisms including *C. glutamicum* was constructed using the Clustal W program (Fig. 7). As expected, NDH-2 of *Corynebacterium* sp. as well as its counterpart *Mycobacterium* sp. were found to be more closely related to eukaryotic enzymes than to other prokaryotic ones. Thus NDH-2 of *C. glutamicum* appears to have evolved from the same ancestor as the enzymes found in yeast and fungi.
NDH-2 of *C. glutamicum* donates electrons to menaquinone at least when it reacts with NADH, while it might preferentially transfer electrons to oxygen with NADPH concomitant with the production of superoxide or hydrogen peroxide. *C. glutamicum* cells contain a relatively high amount of superoxide dismutase as well as catalase, and the superoxide dismutase appears to be localized in both the cytoplasm and the periplasm (unpublished data). Thus NDH-2 of *C. glutamicum* might work as an NADH dehydrogenase as a primary dehydrogenase of the NADH oxidase respiratory chain and also as an NADPH oxidase, or partly as an NADH oxidase, not coupled with the respiratory chain. The latter direct oxidation reactions with NDH-2 appear to correspond to non-energy producing bypass electron transfer routes originally considered to be the bypass oxidase system in the respiratory chain of *C. glutamicum*.\(^{20,27}\)

Our knowledge of the function of NDH-2 in *C. glutamicum* and other organisms is still limited. However, our study together with others will help us to understand the biology of the utilization of NAD(P)H within the cell.

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