Rotenone-insensitive internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* mitochondria: the enzyme expressed in *Escherichia coli* acts as a member of the respiratory chain in the host cells

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Abstract The *NDI1* gene encodes the internal rotenone-insensitive NADH-quinone oxidoreductase localized in the inner mitochondrial membranes of *Saccharomyces cerevisiae*. The T7 tag-fused mature NDI1 was overexpressed in *Escherichia coli*. The overexpressed NDI1 was exclusively found in the membrane fraction. The NDI1-overexpressed membranes showed significantly increased activities of NADH oxidase and NADH-ubiquinone-1 (UQ_1) reductase when compared with the control membranes. Flavone, which is a specific inhibitor of the *S. cerevisiae* NDI1, inhibited almost completely NADH oxidase and NADH-UQ_1 reductase activities of NDI1-overexpressed membranes but scarcely inhibited these activities of the control membranes. In addition, the NADH oxidase activity of the NDI1-overexpressed membranes was also inhibited by KCN as well as the control membranes. These results indicate that the overexpressed NDI1 worked as a member of the respiratory chain in the host cells, even though *E. coli* membranes are different from *S. cerevisiae* inner mitochondrial membranes in terms of quinones and lipid composition.

Key words: NADH-quinone oxidoreductase; Expression; *Saccharomyces cerevisiae*; *Escherichia coli*; Mitochondrion

1. Introduction

Mammalian mitochondria are believed to contain only complex I as the NADH-quinone (Q) oxidoreductase [1–4]. In contrast, mitochondria of *Saccharomyces cerevisiae* have rotenone-insensitive NADH-Q oxidoreductases but lack complex I [5]. In *S. cerevisiae* mitochondria, at least two distinct rotenone-insensitive NADH-Q oxidoreductases are considered to be present. One NADH-Q oxidoreductase is directed to the cytoplasmic phase and the other is directed to the matrix phase (designated internal rotenone-insensitive NADH-Q oxidoreductase) [6]. The internal rotenone-insensitive NADH-Q oxidoreductases (NDI1) of mitochondria are reported to be composed of a single subunit and to contain non-covalently bound FAD as cofactors and no iron-sulfur clusters [2,4,6].

The functional overexpression of mitochondrial oxidative phosphorylation enzyme complexes in bacteria is useful for studies of structure and function of these enzyme complexes. However, to date, such functional overexpression has not been reported. Except for NDI1, other mitochondrial enzyme complexes involved in oxidative phosphorylation are composed of multiple different subunits and contain several different cofactors. On the other hand, as described above, NDI1 is composed of a single subunit and contains FAD only. Therefore, it is of interest to attempt to natively overexpress the *NDI1* gene in *Escherichia coli*.

2. Materials and methods

A 5.5 kbp *Kpn*I/*Pst*I DNA fragment bearing the full-length *NDI1* was excised from λ 7056 (approximately 17 kbp *S. cerevisiae* DNA inserted) and ligated into *Kpn*I/*Pst*I-cut cloning vector pTZ19U. The resulting plasmid was designated pKP5.5. A 2.3 kbp *Sal*I/*Eco*RV fragment containing the full-length *NDI1* was again excised from pKP5.5 and ligated into *Sal*I/*Sma*I-cut cloning vector pTZ18U. The resulting plasmid was designated pRVS2.3. Two oligonucleotide primers were designed. One was to generate a *Nde*I recognition site at the precursor protein initiation codon: 5′-GAAACTAAAAACC*CATATG*C-TATCG-3′ (the underlined bases were altered from *S. cerevisiae* DNA, and italic bases indicate the *Nde*I site). The other was to construct a *Bgl*II site at the mature protein initiation codon: 5′-CTTCCACC*AGATCT*ACAGGGGTGG-3′ (the underlined base was mutated from *S. cerevisiae* DNA and italic bases show the *Bgl*II site). Site-specific mutagenesis was carried out using the Bio-Rad in vitro mutagenesis kit. The resulting plasmids were designated pRVS(NdeI) and pRVS(BglII), respectively. pRVS(NdeI) was cut with *Nde*I and *Bcl*I. The *Nde*I/*Bcl*I fragment was ligated into the *Nde*I/*Bam*HI site in pET11a and pET16b. As described previously [7], pET16b was designed for expression of the His_{10} tag-fused protein. The resulting expression plasmids were designated pET11a(NDI1) and pET16b(NDI1), respectively. pRVS(BglII) was digested with *Bgl*II and *Sac*I. The *Bgl*II/*Sac*I fragment was ligated into the *Bam*HI/*Sac*I site in pET24a. pET24a was designed for expression of the T7 tag-fused protein. The resulting expression plasmid was designated pET24a(NDI1-m).

Competent *E. coli* strains BL21(DE3) and BL21(DE3)pLysS were transformed with pET24a(NDI1-m), pET11a(NDI1), and pET16b(NDI1). Although efforts have been made to express the full length of NDI1 using pET11a(NDI1) or pET16b(NDI1), the expression has not been successful as yet. In contrast, T7 tag-fused mature NDI1 was expressed in *E. coli*, using pET24a(NDI1-m). The optimum expression procedure of pET24a(NDI1-m) was as follows. Colonies transformed with pET24a(NDI1-m) lifted from the 2×YT agar plate containing 30 μg/ml kanamycin were grown at 37°C in 10 ml of 2×YT medium plus kanamycin (30 μg/ml) to the stationary phase. This suspension was used to inoculate 500 ml of 2×YT medium (+30 μg/ml kanamycin). Cells were grown by stirring at 70–80 rpm at 37°C to an absorbance of approximately 0.6 at 600 nm. IPTG was then added (final concentration of 1 mM) and the cells were grown by slow gyration (70–80

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Abbreviations: Q, quinone; UQ_1, ubiquinone-1; NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase(s); complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase(s); NDI1, internal rotenone-insensitive NADH-Q oxidoreductase in mitochondria; NDH-2, bacterial NADH-Q oxidoreductase lacking the energy-coupling site; SDS, sodium dodecylsulfate; IPTG, isopropyl-β-D-thiogalactopyranoside; deamino-NADH, reduced nicotinamide hypoxanthine dinucleotide; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; SMP, submitochondrial particles (inside-out membranes); T7 tag-fused mature NDI1, mature NDI1 fused by T7 tag sequence MASMTGGQQMGRG at the N-terminus

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rpm) for 14 h at 37°C. Competent strains *E. coli* BL21(DE3) harboring pET24a and lacking the plasmid were cultured by the same procedure.

Preparation of cytoplasmic and membrane fractions [8], raising antibody to the NDI1 [8], purification of T7-tag fused NDI1 (the protocol from Novagen), protein sequence determination [9], SDS-polyacrylamide gel electrophoresis [10], amino acid composition [11], DNA and amino acid sequence analyses [12], immunoblotting [8], and assays of NADH oxidase, deamino-NADH oxidase, NADH-UQ_1 reductase and deamino-NADH-UQ_1 reductase activities [13–15] were done according to the references cited.

3. Results

We attempted to express the precursor and mature NDI1 in *E. coli* using expression plasmids pET11a(NDI1), pET16b(NDI1) and pET24a(NDI1-m). Only expression of pET24a(NDI1-m) in *E. coli* strain BL21(DE3) was successful. The anti-T7 tag antibody reacted with a single band of $M_r = 57\,000$ in SDS gels of the membrane fraction (but not in the soluble fraction) of transformed BL21(DE3) cells (Fig. 1). The apparent molecular mass of 57 kDa corresponded to the sum of the masses of mature NDI1+T7 tag. Our expressed T7-tagged mature NDI1 was extracted by 0.3% Triton X-100 from the *E. coli* membranes and purified by anti-T7 antibody affinity column (see Fig. 2). Higher Triton X-100 concentrations gave no improvement in yield of the enzyme extraction. The amino acid composition of the expressed T7-tagged mature NDI1 corresponded reasonably well to that deduced from DNA encoding the T7-tagged mature NDI1 (data not shown). In addition, in order to obtain sequence information on the T7-tagged mature NDI1, the purified polypeptide was cleaved by CNBr. One of the CNBr peptides ($M_r = 26\,000$) had an N-terminal sequence of KVIDPQ, which perfectly matched the deduced primary structure at the carboxyl side of M_{43} of the NDI1. These results indicate that the yeast mature NDI1 enzyme was correctly expressed in *E. coli*.

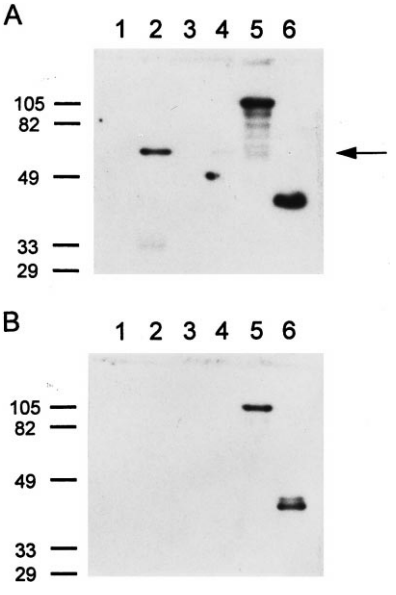

Fig. 1. Western blotting analyses of the expressed mature NDI1 of *S. cerevisiae* using anti T7 antibody. A: Membrane fraction. B: Soluble fraction. 2.5 μg of protein were applied on each lane. Lane 1, BL21(DE3) transformed with pET24a; lane 2, BL21(DE3) transformed with pET24a(NDI1-m); lane 3, BL21(DE3)/pLysS transformed with pET24a; lane 4, BL21(DE3)/pLysS transformed with pET24a(NDI1-m); lane 5, 116 kDa β-galactosidase expressed in BL21(DE3); lane 6, 37 kDa control protein with T7 tag sequence.

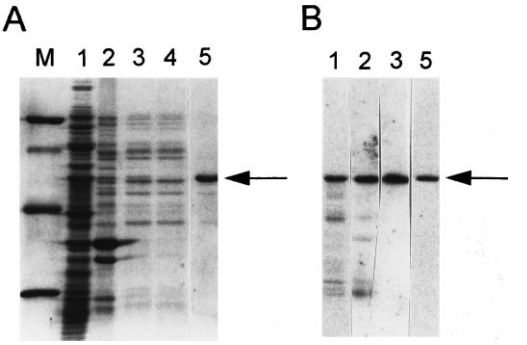

Fig. 2. Purification of the expressed T7 tag-fused mature NDI1 from *E. coli*. A: SDS-polyacrylamide gel. B: Immunoblot using alkaline phosphatase-conjugated monoclonal antibody to the T7 tag. Lane (M), molecular markers used were 97, 64, 45 and 31 kDa; lane 1, cell lysate of *E. coli* harboring pET24a(NDI1-m); lane 2, membrane fraction; lane 3, extract with 0.3% Triton X-100 from membrane fraction; lane 4, fraction was not absorbed by anti-T7 tag antibody affinity column; lane 5, purified T7 tag-fused mature NDI1.

In the preparation step, the anti-T7 tag antibody affinity column turned yellow upon loading the Triton X-100 extract from the membrane fraction, suggesting that the expressed enzyme bears FAD. Therefore, the enzyme assays were carried out with the NDI1-overexpressed membranes in order to assess whether the expressed NDI1 is functionally active in *E. coli* membranes. The NDI1 membranes exhibited approximately 3.5 and 12.5 times, respectively, higher NADH oxidase and NADH-UQ_1 reductase activities than the membranes isolated from *E. coli* harboring pET24a (data not shown). The deamino-NADH oxidase activities were extremely low in all three membranes. Regarding the activity ratio of deamino-NADH oxidase to NADH oxidase the NDI1-overexpressed membranes showed a significantly higher value than the other two control membranes. In the case of the membrane-bound NDH-2 of *E. coli*, the ratio was estimated to be 0.007. A similar ratio was obtained from membrane-bound NDH-2 in *Bacillus subtilis*. On the other hand, the ratio was 0.08 using *S. cerevisiae* SMP. This value is similar to the ratio of the NDI1-overexpressed membranes. DCCD treatment (at 520 μM for 1 h at 20°C) did not affect the NADH-UQ_1 reductase activity of the NDI1-overexpressed membranes, whereas the activities of the control membranes were inhibited by 50% by treatment with DCCD. As reported previously [14], DCCD scarcely affects NADH-UQ_1 reductase activity of the *S. cerevisiae* SMP. On the other hand, DCCD treatment inhibits NADH-UQ_1 and NADH-$K_3Fe(CN)_6$ reductase activities of the NDH-2-overexpressed *E. coli* (IY-91) membranes by approximately 50% (T. Yagi, unpublished results). In addition, the ratio of NADH-UQ_1 reductase to NADH oxidase in NDI1-expressed membranes was high (approximately 6). Similar trends were observed in *S. cerevisiae* SMP but not in *E. coli* membranes (approximately 1.5). Taken together, these results suggest that the T7 tag-fused mature NDI1 was functionally overexpressed in *E. coli* membranes.

In order to confirm the functional expression of the mature NDI1, the effect of flavone, a specific inhibitor for *S. cerevi-*

Table 1
Comparison of kinetic parameters of NADH oxidase among NDI1-overexpressed and control membranes with and without pET24a[a]

	Membranes		
	NDI1	+pET24a	−pET24a
V^{b}	8.8	2	3.2
K_m^{NADH} (μM)	8.3	23	25
V/K_m^{NADH}	1.06	0.09	0.13

[a]Assay conditions are described in Section 2.
[b]Micromol of NADH oxidized per min per mg of protein.

siae NDI1 [6], was investigated on the NADH oxidase of the NDI1-expressed membranes. As shown in Fig. 3, NADH oxidase of the NDI1-expressed membranes is sensitive to flavone as opposed to the activities of the control membranes. The I_{50} value of flavone in NDI1 membranes is approximately 100 μM, which is consistent with the I_{50} value (95 μM) of the membrane-bound and isolated yeast NDI1 [6]. A similar I_{50} value (90 μM) was found for the NADH-UQ_1 reductase activity of the NDI1-overexpressed membranes.

The respiratory chain in aerobically grown *E. coli* is composed of NADH-Q oxidoreductases (NDH-1 [FMN and several iron-sulfur clusters] and NDH-2 [FAD]), succinate-Q oxidoreductase (FAD and FeS clusters), quinones (ubiquinone-8, menaquinone-8, and demethylmenaquinone-8), and two quinol oxidases [16,17]. On the other hand, the respiratory chain in *S. cerevisiae* mitochondria contains NADH-Q oxidoreductases (NDI1 [FAD] and external NDH), succinate-Q oxidoreductase, ubiquinone-6, complex III (hemes b_H, b_L and c_1 and [2Fe-2S]), and cytochrome oxidase (3Cu and hemes *a* and a_3). The inhibitory effect of flavone in Fig. 3 suggests that the NADH oxidase of the NDI1-overexpressed *E. coli* membranes is almost entirely catalyzed by the expressed yeast NDI1. The results with deamino-NADH and DCCD support this notion. If the expressed NDI1 plays a role as a member of the host cell respiratory chain, the NADH oxidase catalyzed by the NDI1 should be inhibited by specific inhibitors for other respiratory chain enzyme complexes in host cells in a similar manner to the control membranes. KCN is recognized

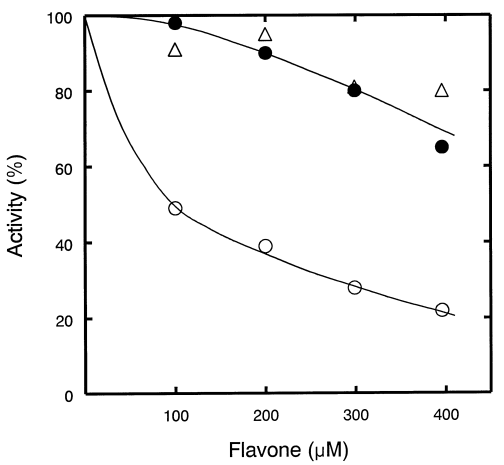

Fig. 3. Effect of flavone on NADH oxidase activities of membranes from NDI1-overexpressed BL21(DE3) (○), BL21(DE3) transformed with pET24a (●), and BL21(DE3) (△). The assays were performed at membrane concentrations of 5 μg of protein/ml at 30°C.

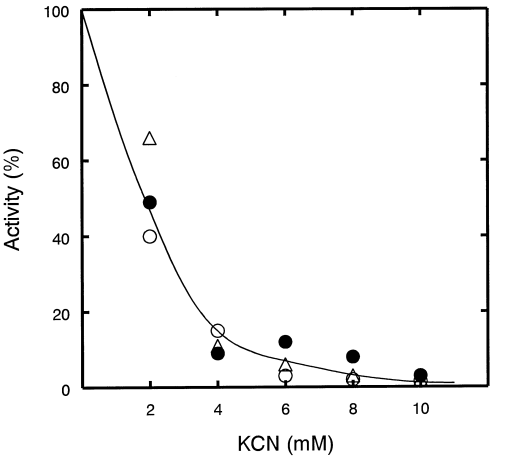

Fig. 4. Effect of KCN on NADH oxidase activities of membranes from NDI1-overexpressed BL21(DE3) (○), BL21(DE3) transformed with pET24a (●), and BL21(DE3) (△). For the assays, the membranes were added to the reaction mixture to a final concentration of 5 μg of protein/ml.

to be a specific inhibitor of the quinol oxidase enzyme complexes in the *E. coli* respiratory chain [16]. We investigated the effect of KCN on the NADH oxidase activities of NDI1-overexpressed membranes and control membranes in order to verify this issue. As shown in Fig. 4, the inhibitory effects of KCN on the NADH oxidase of various membranes are similar to each other. These results indicate that the expressed NDI1 in *E. coli* membranes functions as a member of the respiratory chain in host cells.

The kinetics of NADH oxidase of the three membranes was reasonably fitted by a simple Michaelis-Menten equation in a range of NADH concentration from 8 μM to 150 μM (data not shown). As seen in Table 1, the K_m value (8.3 μM) for NADH in the NDI1-overexpressed membranes was approximately three times lower than those in the control membranes (+pET24a and −pET24a) and was also four times lower than the K_m value (31 μM) for NADH for the NDI1 isolated from yeast SMP [6]. Concerning V/K_m, which is equivalent to the first-order rate constant for NADH binding to the NADH dehydrogenase, the NDI1-overexpressed membranes exhibit a value one order of magnitude higher than the two control membranes. These results suggest that the NDI1 expressed in *E. coli* oxidizes NADH more efficiently than its authentic NDH-2.

4. Discussion

Although inner yeast mitochondrial membranes are significantly different from *E. coli* cytoplasmic membranes in terms of quinones and lipid composition [6,17], *S. cerevisiae* NDI1 can be functionally overexpressed in *E. coli* and acts as a member of the respiratory chain in host cells. Therefore, the functional expression of mitochondrial multiple subunit enzyme complexes involved in oxidative phosphorylation in *E. coli* may be possible although several problems should be overcome.

Mammalian mitochondria oxidize NADH only through complex I. As far as we know at present, complex I has the most intricate structure among the membrane-bound enzyme

complexes (at least 43 unlike subunits) [1–3]. Recently, it has been reported that defects of complex I are involved in many human mitochondrial diseases [18]. Various chemotherapies have been reported to be ineffective at the present time [19]. Dysfunction of complex I in human mitochondria causes three problems: (a) decrease in ATP synthesis, (b) increase in the ratio of NADH to NAD, (c) production of superoxide anion. The impairment of proton pumping by one of the three proton translocation sites does not appear to be as severe a health hazard as the inability of the mitochondria to oxidize NADH. Therefore, a possible approach for coping with complex I defects is to introduce into mammalian mitochondria a NDI1-type enzyme. As described above, NDI1 is a versatile enzyme. Therefore, it may be conceivable that NDI1 is an excellent candidate for this purpose at the present time.

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