Roles of subunit NuoL in the proton pumping coupling mechanism of NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli*

Received January 29, 2016; accepted March 9, 2016; published online April 26, 2016

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Respiratory complex I has an L-shaped structure formed by the hydrophilic arm responsible for electron transfer and the membrane arm that contains protons pumping machinery. Here, to gain mechanistic insights into the role of subunit NuoL, we investigated the effects of Mg^{2+} , Zn^{2+} and the Na^+/H^+ antiporter inhibitor 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) on proton pumping activities of various isolated NuoL mutant complex I after reconstitution into *Escherichia coli* double knockout (DKO) membrane vesicles lacking complex I and the NADH dehydrogenase type 2. We found that Mg^{2+} was critical for proton pumping activity of complex I. At 2 μM Zn^{2+} , proton pumping of the wild-type was selectively inhibited without affecting electron transfer; no inhibition in proton pumping of D178N and D400A was observed, suggesting the involvement of these residues in Zn^{2+} binding. Fifteen micromolar of EIPA caused up to ~40% decrease in the proton pumping activity of the wild-type, D303A and D400A/E, whereas no significant change was detected in D178N, indicating its possible involvement in the EIPA binding. Furthermore, when menaquinone-rich DKO membranes were used, the proton pumping efficiency in the wild-type was decreased significantly (~50%) compared with NuoL mutants strongly suggesting that NuoL is involved in the high efficiency pumping mechanism in complex I.

Keywords: complex I/energy coupling/menaquinone/proton pumping/ubiquinone.

Abbreviations: DDM, dodecyl- β -D-maltoside; DKO, double knockout; dNADH, deamino-NADH; DQ, decylubiquinone; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; HAR, hexaammineruthenium; MK, menaquinone; Mnh, multisubunit Na^+/H^+ antiporter; Mrp, multiple resistance and pH adaptation antiporter; NDH-2, NADH dehydrogenase type 2; Q, quinone; ROS, reactive oxygen species; SQ, semiquinone; UQ, ubiquinone; WT, wild-type.

Complex I (NADH:quinone oxidoreductase) (E.C.1.6.5.3) is one of the largest multi-subunit membrane protein complex found in mitochondria and many aerobic microorganisms. It plays a central role in cellular energy metabolism, and complex I dysfunction has been implicated in a variety of human mitochondrial diseases including neurodegenerative diseases and aging (1, 2). Complex I transfers two electrons from NADH to ubiquinone (UQ), translocates protons across the membrane, and generates a transmembrane electric potential and proton gradient essential for ATP synthesis and transport processes (3–6). Recent X-ray crystal structures from prokaryotic and eukaryotic complex I confirmed (7–9) that the redox reaction which involves flavin mononucleotide (FMN), a chain of seven Fe/S clusters (4), and UQ, takes place in the hydrophilic peripheral domain of the L-shaped molecule, whereas proton pumping machinery is equipped in the hydrophobic membrane domain far from the catalytic site. The membrane-bound subunits NuoL (*Escherichia coli* name)/ND5, NuoM/ND4 and NuoN/ND2 are related to subunits of bacterial multiple resistance and pH adaptation (Mrp) family of Na^+/H^+ antiporters (10), suggesting some similarities in their mechanisms of ion translocation. It has been hypothesized that long-range conformational change triggered by redox energy drives proton translocation through these antiporter-like subunits. However, the molecular details of how the conformational changes are transmitted into the antiporter domains to activate proton pumps are far from being understood.

We have previously shown the functional importance of NuoL/ND5 for redox-linked proton pumping coupling mechanism by mutagenesis study in the *E. coli* NuoL subunit (11). Although NuoL is situated at the distal end of the membrane domain, specific point mutations in NuoL could change the degree of coupling between electron transfer and proton pumping. Peripheral deamino-NADH (dNADH): $K_3Fe(CN)_6$ reductase activities basically remained unchanged in all the NuoL mutants, whereas the proton pumping efficiency (the ratio of H^+/e^- , the initial pumping rate versus NADH oxidase) was decreased in most NuoL mutants by 30–50%, compared with the wild-type (WT) (11). Especially, the proton pumping efficiency in D178N, D303A and D400A mutants was significantly decreased to 50%, suggesting that the H^+/e^- stoichiometry became half ($2H^+/2e^-$), based on the literature that complex I has the high proton pumping efficiency, a stoichiometry of $4H^+/2e^-$.

In this study, to gain a more detailed mechanistic insight, by using purified the WT and NuoL mutant

complex I, we further investigated how mutations of conserved charged amino acids in the entrance and exit of the proposed proton pumping pathways in NuoL affect the proton-pumping machinery of complex I. To facilitate purification of each mutant, we newly generated each NuoL mutation in a strain (MC4100/*His₉nucE*) containing nine histidine coding sequence inserted upstream of *nucE* in the chromosome (12) by homologous recombination. We have shown that the WT complex I was successfully purified via the His-tag. By using this method, we were able to obtain enough amounts of pure and intact *E. coli* NuoL mutants, regardless of the expression levels of NuoL mutant complex I. Also, we have developed an instant reconstitution method, which is alternative to preparing proteoliposomes (13–16), to measure proton pumping activities of isolated mutant complex I for our screening purpose. Preliminary results (17) showed us the feasibility of reconstituting purified complex I into *E. coli* double knockout (DKO) membrane vesicles. Our *E. coli* DKO membrane vesicles contain neither complex I nor the alternative NADH dehydrogenase (NDH-2), showing no NADH-initiated oxidase and H⁺ pumping activities. Regular traditional preparation of proteoliposomes takes at least 3–4 h, whereas our new membrane reconstitution allows us to analyse proton pumping and NADH oxidase activities of various mutant complex I in 5 min. We found that the results by this DKO reconstitution method qualitatively reflected electron transfer and proton pumping activities of the WT and NuoL mutants, compared with the results with membrane vesicles prepared from the WT and NuoL mutant strains (11).

Using this system, we analysed the effects of inorganic divalent cations, Mg²⁺ and Zn²⁺ on proton pumping activities. Mg²⁺ plays an important role in transmembrane ion movement (18, 19), and Mg²⁺ binding is suggested to have a structural role in the stabilization of the interface between the two subunits important for proton pumping in cytochrome *c* oxidase (20). The Zn²⁺ binding is known to inhibit proton transfer activity in the bacterial reaction centre (21), cytochrome *bcl* complex (22) and cytochrome *c* oxidase (23, 24). Although the effects of Mg²⁺ and Zn²⁺ on electron transfer activities (NADH:decylubiquinone (DQ) or NADH:hexaammineruthenium (HAR) or K₃Fe(CN)₆) have been well studied (13, 25, 26), this study is the first detailed report for their effects on proton pumping activities of complex I. We also studied the effects of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), a specific inhibitor for Na⁺/H⁺ antiporters, and menaquinone (MK), a 'low potential' electron acceptor.

Experimental Procedures

Preparation of the knockout NuoL (Δ NuoL) and mutant strains in the (*His*)₉-*nucE* MC4100 cells

The (*His*)₉-*nucE* MC4100 (*F*[−], *araD139*, Δ (*arg F-lac*)*U169*, *ptsF25*, *relA1*, *flb5301*, *rpsL 150*...) strain was generated previously for efficient purification purpose (12). The Δ NuoL [Δ (*nucL*::*Spc*)] and mutant NuoL strains were generated in this strain by employing the same method described previously (11). The presence of mutation in the genomic gene was verified by PCR and DNA sequencing.

Isolation of complex I

Escherichia coli complex I was isolated from the WT, Δ NuoL and NuoL mutant strains by following the procedure published previously (12). Briefly, complex I was extracted from the membrane fraction with dodecyl- β -D-maltoside (DDM) at a final concentration of 1.2% (w/v), isolated using Ni-NTA resin, desalted, and concentrated to 3–8 mg protein/ml. The purified enzyme was quickly frozen in liquid nitrogen and stored at −80 °C until use.

Generation of DKO *E. coli* strains

There are two types of NADH dehydrogenases in *E. coli* membranes. One is called, complex I (NADH dehydrogenase type 1 or NDH-1), and the other is NDH-2. To eliminate NADH dehydrogenase activities that interfere with purified complex I proteins in our membrane reconstitution experiments, we decided to generate a DKO strain in the *E. coli* MC4100 strain, which lacks both complex I and NDH-2 activities. First, we chose a complex I deficient strain, which was previously generated in our lab by knocking out the iron–sulphur cluster N1b. Cluster N1b is located in the NuoG subunit and essential for electron transfer activity of complex I as well as the complex I assembly. The cluster N1b knockout (Δ N1b strain) was generated by homologous recombination from the *nucG* knockout strain [Δ (*nucG*::*Spc*)] described in ref. (27), in which all four cysteine residues ligating to cluster N1b were replaced with alanine (C36A, C47A, C50A and C69A), using a gene replacement technique with pKO3 (28). The detailed procedures were published elsewhere (27, 29, 30). In this Δ N1b strain, no dehydrogenase subcomplex (NuoEFG subcomplex) of complex I was detected in the membrane and supernatant fractions. Next, the Δ N1b strain was further used to knock out the *ndh* gene (encoding NDH-2), yielding a DKO strain by using the same gene replacement technique as used to generate the Δ N1b strain.

Preparation of *E. coli* DKO membrane vesicles

The standard *E. coli* DKO membrane vesicles were prepared according to (29). In brief, the cells were grown in 250 ml of Terrific Broth medium (31) until A₆₀₀ of ~6 and were then harvested at 5,800 × g for 10 min. The cells were resuspended at 10% (w/v) in a buffer containing 50 mM Bis-Tris (pH 6.0), 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% (w/v) glycerol. The cell suspensions were passed once in a French press at 25,000 p.s.i. and centrifuged at 23,400 × g for 10 min. The supernatant was ultracentrifuged at 256,600 × g for 1 h. The pellet was resuspended in a buffer containing 50 mM Bis-Tris (pH 6.0), 2 mM CaCl₂ and 10% glycerol. The resulting membrane vesicles were frozen in liquid nitrogen and stored in small aliquots at −80 °C until use. To prepare UQ-rich and MK-rich membranes, the cells were grown in 280 ml of Terrific Broth medium in a 2.8-l flask at 250 rpm for 5 h, and in 2.5 l of Terrific Broth medium in a 2.8-l flask at 80 rpm for 12 h, respectively.

Reconstitution of purified complex I into DKO membrane

Purified WT and NuoL mutant complex I were mixed with DKO membrane vesicles by tapping in an Eppendorf tube with a total volume of 50 μ l at 1:20 ratio (typically, 1:20 mg/ml), centrifuged briefly and then placed on ice right before use. We measured proton pumping and NADH oxidase activities in parallel for each experiment.

Proton translocation activity

The generation of a proton gradient was determined by monitoring the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA, Sigma). Reconstituted membranes, 2.5–5 μ g, were added to the assay buffer, 50 mM MOPS pH 7.0 containing 50 mM KCl, 20 mM MgCl₂ and 0.2 μ M ACMA as a standard condition and incubated at 30 °C for 3 min. In the experiments to investigate the effect of MgCl₂ on proton pumping activities, we varied MgCl₂ from 0 to 20 mM. The fluorescence was detected with a Fluoromax-4 spectrofluorometer (Horiba) at an excitation wavelength of 430 nm and an emission wavelength of 480 nm. The reaction was started by the addition of 150 μ M NADH. The slopes for the initial acidification rates were calculated from the data using a 5-s time window 5 s after the addition of NADH.

Fig. 1 Proton pumping (A) and NADH-oxidase (B) activities of the WT complex I reconstituted in DKO membranes. The traces from 1 through 5 refer to different WT complex I concentrations in the assay mixture: 0.625, 1.25, 2.5, 3.75 and 5 µg/ml, respectively. The insets in (A) and (B) show the relationship between WT complex I concentrations versus initial acidification rate (IAR) or specific NADH-oxidase activities, respectively. All measurements were carried out in the reaction mixture containing 50 mM MOPS/Na pH 7.0, 50 mM KCl, 20 mM MgCl₂, 0.2 µM ACMA and 20 µM DQ. The reaction was initiated by the addition of 150 µM NADH. Both proton pumping and NADH-oxidase activities were measured by using the same reconstituted samples. Averages and standard deviations were calculated from four trials.

Other analytical procedures

NADH-oxidase activity was measured by adding 1–5 µg of reconstituted membranes in the same assay buffer used for proton translocation activity and incubated at 30 °C for 3 min. The absorbance was measured at 340 nm using a Cary 60 spectrophotometer (Agilent). The reaction was initiated by the addition of 150 µM NADH. Extinction coefficient of $\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ for NADH was used for activity calculations. Reported values are the average of three measurements. The extraction and quantification of quinones in DKO membrane vesicles were performed as described in Ref. (12).

Results

Instant reconstitution of purified complex I into *E. coli* DKO membrane vesicles

Proteoliposome preparation requires a relatively long processing time to remove detergent molecules effectively (13–16). In addition, the reproducibility of proteoliposome preparations is rather low in terms of both quality and quantity, even though the same lot of proteins and lipids are used. Thus, in this study, we have developed a simple and efficient screening method for proton pumping and electron transfer assay. We prepared membrane vesicles from a DKO *E. coli* cells, which do not contain both complex I and NDH-2 (32). The DKO membrane vesicles showed no NADH-oxidase activities or NADH-initiated proton pumping activities (32). Through optimization experiments, we found that just briefly mixing by tapping is enough to reconstitute DKO membranes with isolated complex I. The ratio of 1:20 between complex I and DKO membranes typically gave the highest activity per milligram of complex I. Higher detergent (DDM) concentrations (>0.05%) inhibited the activities. Figure 1A shows proton pumping activities of the isolated WT complex I after the instant DKO reconstitution. By using ACMA as a delta-pH indicator, reproducible NADH-linked proton pumping activity was observed after the addition of NADH. The initial rate of proton pumping activities was dose-dependent up to 3.75 µg of DKO reconstituted WT *E. coli* complex I (Fig. 1A, inset). Similarly, the electron transfer activity of DKO reconstituted WT complex I was also

linear up to 3.75 µg of the WT complex I (Fig. 1B, inset). The specific NADH-oxidase activity was in the similar range of the specific NADH:DQ activities obtained from proteoliposome experiments, around 4–6 µmol/min/mg. Therefore, we set the amount of complex I at 2.5 µg/ml for further assays under various conditions. As both proton pumping and NADH-oxidase activities require complex I to be successfully inserted into the membrane, the presence of these activities strongly suggests that a substantial population was inserted in the right orientation. In fact, there was no difference in NADH:ferricyanide activities of these DKO membranes reconstituted with the WT complex I in the presence or absence of 1% DDM. This indicates that we can rule out the possibility that some population of complex I was inserted in the wrong direction, which usually happens for proteoliposomes. We also confirmed that the specific *E. coli* complex I inhibitor squamotacin completely inhibited the both NADH oxidase and proton pumping activities (data not shown).

Purification of NuoL mutant complex I

Sequence comparisons previously suggested that NuoL/ND5 of complex I bears striking resemblance to MrpA/MnhA of multisubunit secondary Na⁺/H⁺ antiporters (11). In this study, we introduced NuoL point mutations at highly conserved charged residues E144, D178, D303, H334 and D400 (*E. coli* numbering) in the (*His*)₉-*nucE* MC4100 strain for purification, which are located near the entrances and exits of the proposed proton-pumping pathways (33) as shown in Fig. 2. We purified the total of eight NuoL mutant complex I, E144Q, D178N, D303A, D303N, H334Q, D400A, D400E and D400N. Based on the SDS-PAGE patterns (Fig. 2A), these purified NuoL complex I contain all the subunits and have similar purity. The UV-Vis spectra data (Fig. 2B) suggest that they contain roughly similar amount of cofactors, although a trace amount of cytochrome b impurity was noticeable in H334Q and D178N.

Fig. 2 (A) SDS-PAGE analysis and (B) UV-visible absorption spectra of purified WT and various NuoL mutant proteins. Samples (10 μ g) were loaded onto a 10% Tris-Tricine SDS-PAGE gel. lane 1, WT; lane 2, E144Q; lane 3, D178N; lane 4, D303A; lane 5, D303N; lane 6, H334Q; lane 7, D400A; lane 8, D400E; lane 9, D400N. The UV-visible spectra were taken in 50 mM Bis-Tris (pH 6.0) containing 10% glycerol. All the data were normalized at 1 mg/ml.

Fig. 3 Effects of Mg^{2+} on proton-pumping and electron transfer activities in the WT and various NuoL mutant complex I reconstituted into DKO membranes. Initial acidification rates (A) and NADH-oxidase activities (B) of at various Mg^{2+} concentrations are shown. Values are means $\pm$ SD ($n = 3-4$). (C) The locations of the mutated amino acids are shown in the NuoL subunit structure extracted from the three-dimensional crystal structure of *Escherichia coli* complex I (PDBID: 3RKO).

Mg^{2+} plays a critical role in operating proton pumping machinery of complex I

Using this instant reconstitution method with DKO membranes, we first studied effects of Mg^{2+} on complex I activities. Surprisingly, there were almost no proton pumping activities (measured as initial acidification rates) detected in the absence of Mg^{2+} in the WT complex I, and proton pumping activities increased in a dose-dependent manner as the concentration of Mg^{2+} was raised up to 10–20 mM (Fig. 3A). Basically, the similar activation effect by Mg^{2+} on proton pumping rates was also observed in all NuoL mutant complex I tested including E144Q, in which it has previously been reported that the proton pumping activity (and dNADH oxidase activities) was drastically decreased to $\sim 10-20\%$ of WT using intact membrane vesicles (11). These data suggest that in order to obtain the maximum proton pumping activities of

complex I, at least 10–20 mM Mg^{2+} was required regardless of point mutation sites (Fig. 3C). Interestingly, however, electron transfer (NADH-oxidase) activities were found to be independent of Mg^{2+} concentrations and were not affected by much except in the D400A mutant, in which the electron transfer activity was almost doubled at 20 mM compared with 0 mM (Fig. 3B). These results suggest that Mg^{2+} plays a crucial role in proton pumping function by complex I. In striking contrast, it does not seem to play an essential role in electron transfer activities of *E. coli* complex I.

Effects of low Zn^{2+} concentrations and EIPA on proton pumping activities of complex I

Next, we studied the effects of Zn^{2+} on complex I activities. It is known that Zn^{2+} rapidly and tightly binds to the resting state(s) of complex I (25).

Table I. Electron transfer activities (NADH-oxidase) of WT and various NuoL mutants in the absence and presence of 2 μ M ZnCl₂ ($n = 3-4$)

	Control	2 μ M ZnCl ₂	<i>P</i> values
WT	4.62 $\pm$ 0.37	4.52 $\pm$ 0.51	0.7738
E144Q	0.41 $\pm$ 0.07	0.52 $\pm$ 0.23	0.4682
D178N*	3.54 $\pm$ 0.27	2.90 $\pm$ 0.14	0.009
D303A*	2.91 $\pm$ 0.60	1.90 $\pm$ 0.38	0.0294
H334Q*	2.37 $\pm$ 0.83	1.29 $\pm$ 0.15	0.0429
D400A*	3.86 $\pm$ 0.04	2.99 $\pm$ 0.57	0.0497
D400E*	2.22 $\pm$ 0.44	1.25 $\pm$ 0.41	0.018
D400N	1.30 $\pm$ 0.27	0.85 $\pm$ 0.29	0.0635

*Difference with respect to the control is statistically significant ($P < 0.05$).

Table II. Electron transfer activities (NADH-oxidase) of WT and various NuoL mutants in the absence and presence of 15 μ M EIPA ($n = 3-4$)

	Control	15 μ M EIPA	<i>P</i> values
WT	4.54 $\pm$ 0.14	4.75 $\pm$ 0.24	0.1814
E144Q	0.47 $\pm$ 0.03	0.54 $\pm$ 0.08	0.2289
D178N*	2.90 $\pm$ 0.60	4.06 $\pm$ 0.52	0.0445
D303A*	3.30 $\pm$ 0.72	2.08 $\pm$ 0.15	0.0193
D303N	1.90 $\pm$ 0.76	1.97 $\pm$ 0.37	0.8739
H334Q*	2.16 $\pm$ 0.14	3.15 $\pm$ 0.48	0.0265
D400A*	2.84 $\pm$ 0.44	3.96 $\pm$ 0.58	0.0217
D400E	1.59 $\pm$ 0.21	1.71 $\pm$ 0.34	0.5701
D400N*	2.10 $\pm$ 0.08	2.80 $\pm$ 0.45	0.0221

*Difference with respect to the control is statistically significant ($P < 0.05$).

Fig. 4 Effects of Zn²⁺ (A) and EIPA (B) on proton-pumping activities in the WT and various NuoL mutant complex I reconstituted into DKO membrane vesicles. Initial acidification rates of proton pumping activities were measured in the absence (solid bars) and presence (open bars) of 2 μ M ZnCl₂ (A) or 15 μ M EIPA (B). Values are means $\pm$ SD ($n = 3-4$).

Reported IC₅₀ values for the Zn²⁺ inhibition of electron transfer activities by complex I are 10–50 μ M (25, 26). As we would like to selectively detect effects of Zn²⁺ on proton pumping activities, we checked the effect of 10 μ M or lower Zn²⁺ concentrations in our system and decided to use 2 μ M Zn²⁺ for comparison between the WT and NuoL mutants. At this concentration, no inhibition of NADH-oxidase activity by Zn²⁺ was observed (Table I), whereas proton pumping activity significantly decreased by 24% (Fig. 4A). The similar inhibitory effect of 2 μ M Zn²⁺ on proton pumping activities was observed in the D303A and D400E complex I. In contrast, the inhibitory effect of 2 μ M Zn²⁺ on proton pumping activities likely disappeared in the E144Q, D178N, H334Q, D400A and D400N (Fig. 4A). The contrasting results between D400A/D400N and D400E seem to suggest that D400 could be involved in a Zn²⁺ binding site.

EIPA has been known as a specific inhibitor for Na⁺-related transporters such as Na⁺/H⁺ exchangers and Na⁺/K⁺ ATPase (34). It was previously reported that EIPA does not efficiently inhibit NADH-oxidase activity of *E. coli* complex I in Δ ndh membranes

(IC₅₀ > 100 μ M) (35) or NADH:UQ reductase activity of the reconstituted purified *E. coli* complex I (14). However, we previously reported that lower concentrations of EIPA inhibit proton pumping activity of the WT complex I but not certain NuoL mutants including D178N from *E. coli* without affecting electron transfer activities in membrane vesicle experiments (11). Thus, we predicted that EIPA might primarily inhibit proton transfer in NuoL and investigated the effects of EIPA on proton pumping activities in the DKO membranes reconstituted with isolated NuoL mutants. In WT, the proton pumping activity was significantly decreased to 60.8% in the presence of 15 μ M EIPA (Fig. 4B), where its NADH oxidase activity was not inhibited (Table II). This result can be interpreted as only a proton transfer pathway being blocked without affecting electron transfer pathway. In contrast, no inhibitory effect of EIPA on proton pumping activity was observed in D178N (Fig. 4B). This result is consistent with our previous report using intact membrane vesicles from the D178N strain (11). Otherwise, proton pumping activities in most NuoL mutants were inhibited by 15 μ M EIPA (Fig. 4B).

Characterization of DKO membranes reconstituted with WT complex I

Our results above support that the instant DKO membrane reconstitution method is useful for the functional assessment of complex I and particularly for screening purposes to compare the proton pumping activities between the WT and NuoL mutants. However, in contrast to proteoliposomes, we need to be more careful about the interpretation of the results, as DKO membranes contain other proteins that might affect our results to some extent. Thus, we further studied some characteristics of our DKO membranes. *Escherichia coli* membranes contain quinol oxidase(s), cytochrome *bo*₃ or/and *bd*, both proton translocating enzymes, which can oxidize the UQ reduced by complex I. First, we checked the effect of NaCN, an inhibitor for quinol oxidases. Proton pumping activities in the DKO membranes reconstituted with WT were strongly inhibited by NaCN regardless of the presence of externally added DQ (Fig. 5A). This is indeed very consistent with our previous observations that proton pumping activity of complex I is very sensitive to

Fig. 5 Effects of NaCN (A), oligomycin (B) and various counter anions of magnesium salts (C) on proton-pumping activities of DKO membrane reconstituted with WT complex I. (A) Proton-pumping activity of WT-DKO membrane was measured in the presence of 20 μ M DQ, 150 μ M NADH and various NaCN concentrations. Trace 1 represents the control with no NaCN. Traces 2 through 7 represent NaCN concentrations at 2, 4, 6, 8, 10 and 15 mM, respectively. (B) Proton-pumping activity of WT-DKO membrane was measured in the presence of 20 μ M DQ, 150 μ M NADH and various oligomycin at 0, 5, 10 and 15 μ g/ml. Traces 1–4 correspond increasing oligomycin concentrations. The plots of amplitudes of acidification (ΔF) versus NaCN or oligomycin concentrations are shown in the insets in Fig. 4A and B, respectively. (C) Proton-pumping activity (IAR, initial acidification rate) of WT-DKO membrane was measured in 20 mM of the various magnesium salts. Averages and standard deviations were calculated from four trials. The asterisk symbols on the top of the bars indicate that the difference with respect to the control is statistically significant ($P < 0.05$).

quinol, which we and others (13) previously observed with intact WT membrane vesicles. Probably, rapid accumulation of quinol caused by the inhibition of quinol oxidase reverses the complex I reaction backward, as it is known that complex I catalyse the $\Delta\mu\text{H}^+$ -dependent NAD^+ reductase reaction (36). Next, as the rate of proton pumping could be underestimated by ATP synthase, which decreases ΔpH , we investigated the effect of oligomycin, an inhibitor for ATP synthase. To our surprise, oligomycin inhibited NADH-initiated proton pumping activities by up to 50% (Fig. 5B). Interestingly, we observed that oligomycin also inhibited on quinol-initiated proton pumping activities (by quinol oxidase) in the DKO membrane itself (data not shown). Third, it is possible that the Mg^{2+} effect shown in Fig. 2 could be the result of the increase in chloride added with Mg^{2+} rather than Mg^{2+} itself. Thus, we measured proton pumping activities with other magnesium salts (Fig. 5C). Compared with the chloride, sulphate and nitrate salts gave slightly lower proton pumping and electron transfer activities. Seemingly, the effect of anions on proton pumping is likely through the decreasing effect of electron transfer activities. This confirms that the increase observed in proton pumping activities with increasing MgCl_2 concentrations (Fig. 3A) is solely due to Mg^{2+} .

Proton pumping and electron transfer activities in UQ-rich and MK-rich DKO membranes reconstituted with *E. coli* complex I

MK is known to work as an electron acceptor in *E. coli* anaerobic respiratory chain. To investigate how redox potential difference between UQ and MK affects proton pumping activities of complex I, we prepared

UQ-rich and MK-rich DKO membrane vesicles from *E. coli* cells grown aerobically and anaerobically, respectively. Our membrane vesicles contained $\sim 70 \mu\text{mol}$ of total quinone per mg of membranes, and typically, MK was 2.5 times higher than UQ in MK-rich membranes, UQ was 2.5 times higher than MK in UQ-rich membranes. We found that the initial proton pumping rate was almost half when the WT complex I was reconstituted into MK-rich DKO membrane vesicles compared with that with UQ-rich membrane vesicles (Fig. 6A). In contrast, no significant difference was observed in initial proton pumping activities between the UQ-rich and MK-rich DKO membranes reconstituted with the D178N complex I (Fig. 6B). Interestingly, we noticed that electron transfer activities were always the same or even higher with MK-rich DKO membranes vesicles, indicating somewhat more effective insertion of complex I into a lipid bilayer with MK-rich DKO membranes vesicles. Thus, we repeated at least three different sets of experiments (Table III) and calculated the coupling efficiency ratio after dividing initial proton pumping rates by electron transfer activities for each experiment (Table I), to normalize deviations derived from DKO reconstitution efficiency between UQ-rich and MK-rich DKO membrane vesicles. The efficiency of electron transfer coupling to proton pumping of the WT reconstituted into MK-rich membrane vesicles decreased to 54.9% compared with that with UQ-rich membranes, whereas the coupling efficiency of D178N reconstituted into MK-rich membranes was retained similar to that with UQ-rich membranes (Fig. 6C). The coupling efficiency of other NuoL mutants H334Q and D400A decreased to $\sim 75\%$ (Fig. 6C). These data suggest that MK might be insufficient to induce

Fig. 6 Proton pumping activities of the WT and selected NuoL mutant complex I reconstituted in UQ-rich and MK-rich DKO membranes. UQ-rich and MK-rich DKO membranes were prepared from *E. coli* cells cultured under our regular aerobic and semi-anaerobic conditions, respectively. Representative data of proton pumping traces for the WT (A) and D178N mutant (B) are shown. (C) Percentages of proton pumping efficiency in MK-rich versus UQ-rich DKO membranes calculated from the average of three separate experiments is shown. For each experiment, we carried out at least three measurements ($n = 3-5$). (D) Effect of EIPA on proton pumping activities of the WT complex I reconstituted UQ-rich and MK-rich DKO membranes. The data were obtained from three separate experiments as described in (C); 15 μ M EIPA was used. The asterisk symbols on the top of the bars indicate that the difference with respect to the control is statistically significant ($P < 0.05$).

conformational changes needed for a full operating mode of proton pumping machinery, leading to a lower proton/electron coupling efficiency in WT. However, D178N already lost the high coupling efficiency part of proton pumping operating mechanism, thus, no difference was observed between the coupling efficiency between UQ-rich and MK-rich DKO membranes. As it was previously suggested that low concentrations of EIPA lowers a high coupling efficiency mechanism in WT by probably blocking a part of proton pumping pathway (11), we compared the effects of EIPA between the UQ-rich and MK-rich DKO membrane vesicles reconstituted with WT. The coupling efficiency was reduced to 47% in the UQ-rich membranes and 64.5% in the MK-rich membranes by 15 μ M EIPA (Fig. 6D). The effect of EIPA in the MK-rich membranes was significantly smaller, compared with UQ-rich membranes.

Discussion

Investigating the proton pumping mechanism of complex I is one of the most challenging bioenergetic problems, due to the structural/functional complexity and the difficulty of accounting for the exceptionally high proton pumping stoichiometry of $3-4\text{H}^+/2\text{e}^-$ (proton/

electron) (37–41). In this study, we focused on the most distal membrane subunit NuoL/ND5 more than 150 Å away from the quinone reducing site (8). As it is commonly believed that acidic residues are required near the orifice of proton pathways to facilitate proton uptake, we investigated roles of highly conserved charged amino acids in NuoL for proton pumping mechanism of *E. coli* complex I, using isolated NuoL mutant complex I. To monitor proton pumping activities of isolated complex I, purified proteins have to be reconstituted into proteoliposomes. However, preparing proteoliposomes requires relatively large amount of purified proteins and long processing time, and has a problem in reproducibility. Therefore, we first developed an alternative and convenient method, *in vitro* reconstitution using *E. coli* DKO membrane vesicles, which have no NADH-initiated proton pumping activities or NADH oxidase activities. This membrane reconstitution method allows us to analyse proton pumping activities of various mutant complex I faster (5 min versus 3–4 h) and with less protein (50 versus 250 μ g) than the traditional proteoliposome method. The additional advantage of using DKO membrane vesicles is that DKO membrane preserves natural membrane conditions such as containing native *E. coli* lipids

Table III. Proton pumping and NADH-oxidase activities of the NuoL mutant complex I reconstituted into UQ- and MK-rich DKO membranes

Sample	Trial	UQ-rich DKO membranes			MK-rich DKO membranes			
		Initial acidification rate (IAR)	NADH-oxidase activity (NOA)	IAR/NOA	Initial acidification rate (IAR)	NADH-Oxidase activity (NOA)	IAR/NOA	% (Menaq/Ubiq)
WT	1	3,474.9	3.28	1,058.2	2,156.9	4.28	503.5	47.6
	2	2,682.9	5.01	535.9	1,748.1	5.66	308.6	57.6
	3	4,139.2	3.97	1,043.2	3,313.1	5.35	619.5	59.4
D178N	1	2,491.4	3.06	814.7	2,541.9	2.98	854.2	104.8
	2	2,529.7	4.46	566.6	2,756.0	4.71	585.2	103.3
	3	3,392.0	3.10	1,095.3	3,628.2	4.64	782.2	71.4
H334Q	1	2,095.5	2.94	713.9	2,309.3	4.21	549.0	76.9
	2	1,974.7	2.78	710.1	2,178.0	4.17	521.8	73.5
	3	1,965.4	2.86	686.1	1,901.2	4.20	452.7	66.0
D400A	1	2,456.7	4.19	586.3	2,621.8	5.35	490.1	83.6
	2	2,579.5	4.13	624.6	2,658.6	5.68	468	74.9
	3	2,729.6	4.13	660.9	2,990.7	5.29	565.3	85.5

The values are the average of $n = 3-4$ measurements.

that are necessary for the stabilization and effective insertion of complex I into a lipid bilayer, and for optimal activity. The problem with using DKO membranes could be the interpretations of the results, which could be complicated by other proteins contained such as quinol oxidases and ion transporters. However, as shown in Fig. 1, there is a linear relationship between the amount of complex I and proton pumping and electron transfer activities. Thus, as long as the same lot of DKO membranes is used, these background effects applied to all the samples, thus, qualitative and relative comparisons between the WT and mutants are still possible and valid.

We previously reported that proton pumping activities in membrane vesicles prepared from the E144Q, D178N and D400A strains are ~5%, ~45% and 45% of the WT, respectively (11). However, in the DKO reconstitution experiments, proton pumping activities of E144Q, D178N and D400A are ~35%, ~75% and 85% of the WT, respectively (Fig. 3A). The differences between the WT and mutants are much smaller. This apparent discrepancy could be explained by the followings. First, the amount of complex I present in the membrane vesicles is varied even from the WT strain, because complex I levels really depend on cell growth phases. The highest expression levels are usually seen in early stationary phase, but the specific activity of complex I could still be varied by up to 50% from batch to batch. Usually, mutants grow slower compared with the WT, so it is more difficult to judge the right time to harvest cells. In addition, mutations tend to destabilize the complex I assembly. So, the amounts of fully assembled complex I remained in the membrane are also varied. Therefore, the observed low activities are not necessarily to be interpreted by the direct effect of mutation in the important residues and could be caused simply by low levels of complex I proteins in the membrane. Second, the discrepancy could also originate from differences in the nature of samples between membrane vesicles and our reconstituted DKO membranes. We have recently discovered that the product QH₂ is extremely inhibitory towards complex I reactions, especially the proton pumping

activity. Low concentrations QH₂ (like 3 μ M) can shut down proton pumping activity instantaneously like an uncoupler (unpublished observations). In membrane vesicles, the generated QH₂ by complex I is promptly removed by the quinol oxidase *bo3* in the membrane. However, in the DKO membranes reconstituted with complex I, it seems that the product QH₂ by complex I is not efficiently consumed by *bo3*. Because, the DKO membranes themselves have shown very strong proton pumping activity by *bo3* after the addition of 6 μ M of QH₂, and this activity can be completely inhibited in the presence of NaCN (data not shown). Compared with this proton pumping activity by *bo3*, NADH-initiated proton pumping activity by complex I in DKO-WT reconstituted membranes is much slower, suggesting almost no contribution from *bo3* for the NADH-initiated proton pumping activity, although substantial QH₂ is produced during the complex I reaction based on NADH-oxidase activity. Therefore, we speculated that the connection between complex I and *bo3* is very loose unlike intact membrane vesicles. Thus, the initial maximum acidification phase is very short especially for the WT and proton pumping is immediately inhibited by QH₂. We cannot eliminate a possibility that we underestimated the proton pumping activity from the WT, because the WT has the highest complex I activity and generated QH₂ at the fastest, and its proton pumping activity is inhibited faster than those of other mutants. As a result, proton pumping activities of mutant complex I became relatively much higher.

It has been not explicitly but generally noticed that Mg²⁺ is required to measure proton pumping activities. However, this study is the first report on the Mg²⁺ effect on proton pumping function of complex I by varying Mg²⁺ concentrations in the assay buffer. We have observed that 0.4–1 mM Mg²⁺ in the reaction mixture helps measure reproducibly the maximum proton pumping activities in proteoliposomes and/or *E. coli* intact membrane vesicles. However, in the DKO reconstitution system, the effect of Mg²⁺ turned to be drastic. Surprisingly, there was almost no proton

pumping activity detected in the absence of Mg²⁺, and 10–20 mM of Mg²⁺ was necessary to obtain the maximum protonpumping activities. In addition, an interesting thing is that electron transfer activities were not affected by Mg²⁺ concentrations (Fig. 3B). It is known that Mg²⁺ interacts with the head-groups of phospholipids in membranes (42). Also, a bound Mg²⁺ ion found in cytochrome c oxidases was suggested to have a structural role in the stabilization of the interface between Subunits 1 and 2 (20). Therefore, perhaps, Mg²⁺ facilitates complex I self-integration into membrane vesicles by stabilizing and aligning membrane subunits in a right direction to secure proton pathways upon conformational changes triggered by quinone reduction. As there was no difference in the Mg²⁺ effect between the WT and NuoL mutants, it can be suggested that those residues are not directly involved in this Mg²⁺ role.

Zn²⁺ binding may disrupt many different components of the mechanism, including proton transfer pathways (proton transfer to bound quinone or proton translocation). Zn²⁺ is believed to be one of the most potent inhibitor of proton channels. The inhibitory action of Zn²⁺ is derived from the metal ion binding to histidine residues, and also to the thiol group of the cysteine residues (43). In fact, it has been reported that Zn²⁺ does not inhibit NADH oxidation at the flavin site or intramolecular electron transfer in complex I, but it does inhibit proton transfer to bound quinone or proton translocation (25). Although the reported IC₅₀ value for the Zn²⁺ inhibition of NADH:DQ activities in isolated *E. coli* complex I is 50 μM (26), in our study, *E. coli* complex I was much more sensitive to Zn²⁺ after the instant DKO reconstitution. This suggests that when complex I is embedded in the membrane, the conformation of complex I is different compared with the conformation as isolated in solution. At 2 μM, we finally did not see any inhibitory effect of Zn²⁺ on NADH-oxidase activity, whereas Zn²⁺ inhibited proton pumping activity in the WT. The results that D400A and D400N but not D400E was unaffected by 2 μM Zn²⁺ suggest that D400 is possibly involved in a Zn²⁺ binding site that involves proton translocation and could be the exit of proton transfer pathway in NuoL (Fig. 3C). Indeed, our data are consistent with the previous results by FT-IR spectroscopy that carboxylic acid residues in a hydrophobic environment were perturbed by the presence of Zn²⁺, which was assumed to be conserved charged amino acids located in proton pathways (26). Also it is interesting to note that E494, which is located in a close proximity to D400, could help in Zn²⁺ binding.

EIPA, like other amiloride derivatives, has been known to inhibit Na⁺/H⁺ antiporters (34, 44). Previously, we have shown that EIPA inhibits mitochondrial and bacterial complex I, and we have reported that the IC₅₀ values of EIPA for NADH oxidase activities of bovine SMP and *E. coli* NDH-1 are 17 μM and >100 μM, respectively (35). Although *E. coli* complex I is more resistant to EIPA, later, we found that low concentrations of EIPA (50 μM or less) primarily inhibit the proton transfer in NuoL (11). In this study, we observed *E. coli* complex I became more

sensitive to EIPA after reconstitution into DKO membranes also in proteoliposome experiments (MN and ENO, unpublished observation). However, by lowering the EIPA concentration to 15 μM, we were able to confirm that low concentrations of EIPA selectively inhibits only the pumping activity in WT by 40% without affecting the NADH oxidase activity, whereas EIPA had no effect on the proton pumping rate in the D178N mutant that seemingly lost the high affinity EIPA binding site (Fig. 4B). The EIPA-binding site(s) has been expected to be in NuoL/ND5, based on the several facts: (1) high sequence similarity between NuoL/ND5 and MrpA/MnhA, although D178 is only conserved in homologs of NuoL (11); (2) Na⁺ transport by *E. coli* complex I but not by *E. coli* complex I devoid of NuoL (45); (3) heterogeneously over-expressed *E. coli* NuoL subunit is capable of supporting the growth of the ΔMrpA mutant (46); (4) EIPA inhibition of the sodium uptake by C-terminally truncated NuoL reconstituted in proteoliposomes (47); and (5) the blocking effect of EIPA on the ND5 labelling with a fenpyroximate photoaffinity analogue (48). Therefore, all together, our results suggest that D178 could be a possible EIPA binding site, although we cannot rule out the possibility that D178A mutation indirectly affected the EIPA binding site.

One advantage to use DKO membranes in our case was the possibility that we could modify quinone compositions in DKO membranes by changing growth conditions to study MK effects on complex I. It is generally believed that MK can serve as an electron carrier only for backward electron transfer in eukaryotic mitochondria under anaerobic conditions (49). In contrast, for *E. coli* complex I, MK could work as an alternative substrate (50). To gain new insight on how the redox potential difference between UQ and MK affects proton pumping activities, we prepared MK-rich DKO membranes and measured the proton pumping activities in reconstituted DKO membranes with the WT and selected NuoL mutants. As the midpoint redox potential of MK/MKH₂ is –80 mV, much lower than that of UQ/UQH₂ (+110 mV), we hypothesized that we might be able to observe a lower proton pumping coupling efficiency with the ‘low redox potential’ electron acceptor MK, compared with UQ. We successfully observed that the decreased proton pumping efficiency and much less additional inhibitory effect by EIPA in the WT reconstituted with MK-rich DKO membranes (Fig. 6C and D, respectively). Our results strongly suggest that ‘low redox potential’ MK cannot operate the high proton pumping capacity mechanism. This can also be supported by the contrasting result of D178N that no difference in proton pumping coupling efficiency between UQ- and MK-rich membranes was detected (Fig. 6C), as D178N has already lost the high efficiency proton pump coupling mechanism (11). It is tempting to speculate that the redox energy released upon MK reduction with NADH by complex I might not be enough for the full operation of proton pumping machinery. Although we cannot rule out a possibility that some unknown differences in membrane components contributed the difference in proton pumping efficiency between UQ- and

MK-rich DKO membranes, this possible background effects apply to the results with NuoL mutants, which did not display drastic differences. Thus, it is highly likely that the redox energy released upon MK reduction with NADH by complex I might not be enough for the full operation of proton pumping machinery. Previously, based on the report using *E. coli* cells grown anaerobically, the H^+/e^- stoichiometry for bacterial complex I was calculated to be 1.5 (41), whereas it is established that the H^+/e^- stoichiometry of mitochondrial complex I is 2. The lower stoichiometry of *E. coli* complex I in this report might be explained by the involvement of MK in the membranes vesicles they used.

In conclusion, our results strongly suggest that D178 in NuoL plays a key role in the indirect high efficiency proton pumping coupling in complex I. The other conserved residues E144, H334 and D400 also seem to be involved in proton pumping and/or coupling mechanisms, as local conformational changes triggered by single mutations in these residues led to the reduction of proton pumping activities and proton pump coupling efficiency and changed sensitivities towards Zn^{2+} and EIPA. Further studies of the role of conserved residues in NuoL are necessary for the understanding of complex I coupling mechanisms.

Acknowledgements

The authors would like to thank Dr Carolina Sepulveda Nunez for discussion and Ms Diana Orlandi for her technical assistance.

Funding

This work was supported by National Institute of Health General Medicine grant R01GM097409 to E.N.-O. and American Heart Association grant 11SDG5560001 to E.N.-O.

Conflict of Interest

None declared.

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