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Existence of Na^+-translocating NADH-quinone reductase in *Haemophilus influenzae*

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Abstract We previously cloned and sequenced *nqr* operon encoding the Na^+-translocating NADH-quinone reductase (NQR) from the marine bacterium *Vibrio alginolyticus*. A gene cluster very similar to *nqr* operon was found to exist in the genome of *Haemophilus influenzae* Rd. We examined the membrane fraction from *H. influenzae*, and the respiratory chain of *H. influenzae* was found to contain a Na^+-dependent NQR that is essentially identical to those found in the marine *V. alginoltyticus*. These results indicate that quite similar to the salt-loving marine bacteria, the blood-loving *H. influenzae* has a redox-driven Na^+ pump and utilizes Na^+ circulation for energy coupling.

Key words: Na^+ pump; Na^+-translocating NADH-quinone reductase; *Haemophilus influenzae*; *nqr* Operon; Complex I; *Vibrio alginolyticus*

1. Introduction

Gram-negative marine and moderately halophilic bacteria have a unique Na^+-translocating NADH-quinone reductase complex (NQR) as a first segment of the respiratory chain [1–5]. The enzyme complex was purified from the marine *Vibrio alginolyticus* and was found to be composed of three subunits, α, β and γ [6]. Recently, we have cloned and sequenced a part of *nqr* operon from *V. alginolyticus* [7,8]. At the same time, Beattie et al. [9] reported the presence of four consecutive open reading frames in the *nqr* operon. In combination with these results, the *nqr* operon was found to be constructed from six consecutive structural genes, where *nqr1*, *nqr3* and *nqr6* corresponded to α-, γ-, and β-subunits, respectively, of the NQR complex. Very recently, Venter and his coworkers [10] reported the complete nucleotide sequence of the genome from the bacterium *Haemophilus influenzae* Rd, and the homology search of *nqr* operon was performed with the whole genome. Surprisingly, a gene cluster almost identical to the *nqr* operon was found to exist in the genome of *H. influenzae*. This organism is not known to have an Na^+-translocating NQR in the respiratory chain. Therefore, we examined the membrane fraction from *H. influenzae* and the respiratory chain of this organism was found to contain an Na^+-translocating NQR, which is very similar to that from the marine *V. alginolyticus*.

2. Materials and methods

2.1. Materials

Ubiquinone-1 (Q-1) was kindly supplied by Eizai Co., Tokyo. *Haemophilus influenzae* Rd, ATCC 51907, was obtained from American Type Culture Collection. Other reagents used were of analytical grade.

2.2. Bacterial growth and preparation of membrane fraction

H. influenzae was shake-cultured at 37°C in a medium containing 3% (w/v) Tryptic soy broth (Difco), Strepto-haemo Supplement (Eiken, Tokyo) and 20 mM potassium phosphate buffer (pH 7.5). Cells were harvested at the late-exponential phase of growth by centrifugation and then washed twice with 10 mM HEPES-Tris (pH 7.5)/5 mM $MgSO_4$. Washed cells were suspended in 10 mM HEPES-Tris (pH 7.5)/5 mM $MgSO_4$/1 mM dithiothreitol/1 mM phenylmethylsulfonylfluoride/10% (w/v) glycerol at a cell density of 130 mg wet weight cells per ml. The suspension was passed through a French pressure cell (Aminco) at 8000 $lbf \cdot in^{-2}$. The membrane fraction was isolated by ultracentrifugation as described in [11], and was finally suspended in 10 mM HEPES-Tris (pH 7.5)/5 mM $MgSO_4$/10% (w/v) glycerol at about 10 mg protein per ml. It was stored at −80°C.

2.3. Enzyme assays

NADH oxidase activity was assayed at 30°C in the reaction mixture containing 25 mM Tris-HCl (pH 7.5), an appropriate concentration of NaCl or KCl, 0.1 mM NADH and the membrane fraction in a total volume of 2.0 ml. The reaction was started by the addition of membrane fraction and the activity was determined from the decrease in absorbance at 340 nm.

The reduction of ubiquinone-1 (Q-1) by NADH was measured at 30°C in the reaction mixture containing 25 mM Tris-HCl (pH 7.5), salt, 0.1 mM NADH, 15 μM Q-1, 10 mM KCN and the membrane fraction. The membranes were preincubated with KCN for 3 min, and then the reaction was started by the addition of Q-1 and NADH. Changes in absorbance difference at the wavelength pair, 242–270.5 nm, were recorded with a Hitachi 557 dual-wavelength spectrophotometer, and the rate of ubiquinol (QH_2) formation was calculated using an absorption coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$. When menadione was used as an electron acceptor, 0.1 mM menadione was added to the reaction mixture and the activity was measured from the decrease in absorbance at 340 nm.

One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH, or the reduction of 1 μmol Q-1 in 1 min.

2.4. Measurement of membrane potential

Generation of a membrane potential, positive inside, in the inverted membrane vesicles was monitored by measuring the absorbance difference of bis(3-propyl-5-oxoisoxazole-4-yl)pentamethine-oxonol (oxonol-VI) at 625–587 nm. The membrane fraction was suspended in a medium containing 25 mM HEPES-Tris (pH 7.5), 10 mM $MgSO_4$, and 0.1 M K_2SO_4 or Na_2SO_4. The suspension was quickly frozen at −70°C, thawed at 30°C, and then briefly sonicated in a bath-type sonicator. This treatment was repeated once. The membrane vesicles loaded with K^+ or Na^+ were used for the measurement of membrane potential. The reaction mixture contained 25 mM HEPES-Tris (pH 7.5), 10 mM $MgSO_4$, 0.1 M K_2SO_4 or Na_2SO_4, 3 μM Oxonol-VI and 1.0 mg protein of the membrane vesicles in a total volume of 2.0 ml. The mixture was incubated at 20°C for 20 min, and then the reaction was started by the addition of 0.1 mM NADH.

2.5. Homology searches

Nucleotide sequences for *nqr1* and *nqr2* were used from the data of Beattie et al. (accession number Z37111) [9], and those for *nqr3*, *-4*, *-5*, and *-6* were from our data (accession number D49364) [8]. Sequence comparisons were performed using the program BLAST to determine

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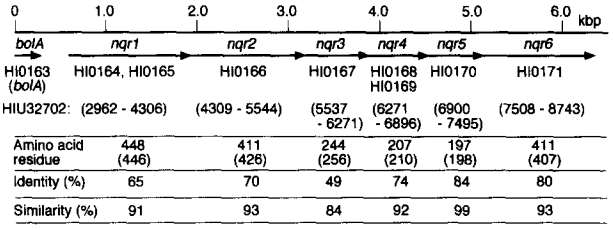

Fig. 1. Comparison of *nqr* operon (*nqr1* to *nqr6*) with a highly homologous region from the genome of *H. influenzae*. In accordance with the sequence of HIU32702, the numbers of nucleotide residues corresponding to each *nqr* gene are shown in parentheses. The predicted number of amino acid residues for each open reading frame is shown together with those for *nqr* gene in the parenthesis.

homology. Sequences were analyzed using the GENETYX-Mac version 7.

3. Results and discussion

3.1. Homology searches of nqr operon

Very recently, Venter and his coworkers [10] reported the complete nucleotide sequence (1 830 137 bp) of the genome from the bacterium *H. influenzae*. We performed the homology search of *nqr* operon with the whole genome of *H. influenzae* and, surprisingly, a region homologous to the *nqr* operon was found in the genome of *H. influenzae*. Identical to the case of *V. alginolyticus*, this region followed to *bolA* gene and the operon was composed of 6 open reading frames each with exactly the same size as that of *nqr* operon from *V. alginolyticus* (Fig. 1). The disagreement in the open reading frames observed in *nqr1*, *nqr4* and *nqr5* could be rearranged by frame shifts. As shown in Fig. 1, the deduced amino acid sequence was more than 65% identical except for Nqr3 with 49% identity. In all structural genes, the sequence similarity attained to well over 84%. Although the functions of this region are totally unknown in *H. influenzae*, these results strongly suggest that the gene cluster from HI0164 to HI0171 constitutes an *nqr* operon encoding Na^+-translocating NADH-quinone reductase. Since *H. influenzae* is not classified into marine and halophilic bacteria, the existence of *nqr* operon is unexpected and it is necessary to verify the expression of Na^+-translocating NQR in this organism.

3.2. Na^+-dependent NADH oxidase in the membrane fraction from H. influenzae

H. influenzae was grown in a rich medium at neutral pH and then the membrane fraction was prepared as described in section 2.2. Fig. 2 shows the effects of NaCl or KCl on the NADH oxidase activity of the membrane fraction. The NADH oxidase activity was greatly stimulated by NaCl, but not by KCl. The concentration of Na^+ required for half-maximum activation ($M_{1/2}$) was estimated to be 5.5±0.6 mM. In the presence of 20 mM KCl, the $M_{1/2}$ for Na^+ decreased to 2.9±0.3 mM. Except for the low values of $M_{1/2}$ for Na^+, these results were essentially similar to those observed with the membrane-bound NADH oxidase from *V. alginolyticus* [12]

The NADH-quinone reductase activity as measured by the formation of ubiquinol from Q-1 was completely dependent on Na^+. Thus, the site of Na^+ activation in the respiratory chain of *H. influenzae* was found to reside in the step of ubiquinone reduction by NADH. When menadione was used as the electron acceptor, the activity was stimulated

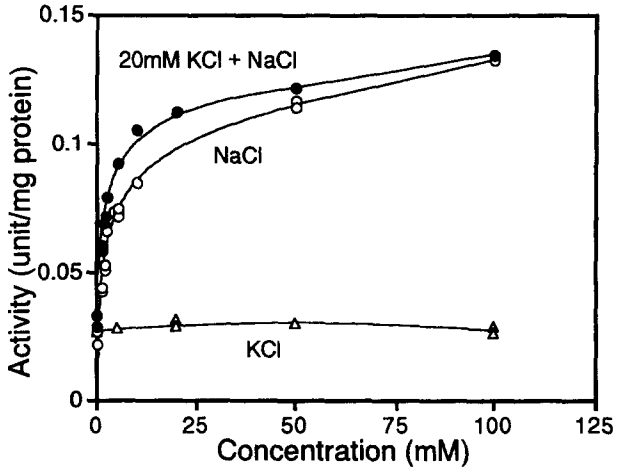

Fig. 2. Effects of NaCl and KCl on the activity of membrane-bound NADH oxidase from *H. influenzae*. The concentration of NaCl or KCl was varied as described in the figure.

about 2-fold by the addition of either 200 mM KCl or NaCl, but no specific requirement for Na^+ was observed in this reaction. These results were in agreement with our previous data that menadione directly interacts with the β-subunit of the NQR complex in *V. alginolyticus*, thereby showing no specific requirement for Na^+ [1,6,13].

3.3. Generation of a membrane potential and effects of inhibitors

The membrane potential generated by Na^+-dependent oxidation of NADH is not completely dissipated by the proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in marine and moderately halophilic bacteria [11]. With the inverted membrane vesicles from *H. influenzae*, Na^+ was required for the generation of membrane potential (Fig. 3). Furthermore, the membrane potential was not completely dissipated by CCCP even by increasing its concentration, and monensin was required for the complete dissipation.

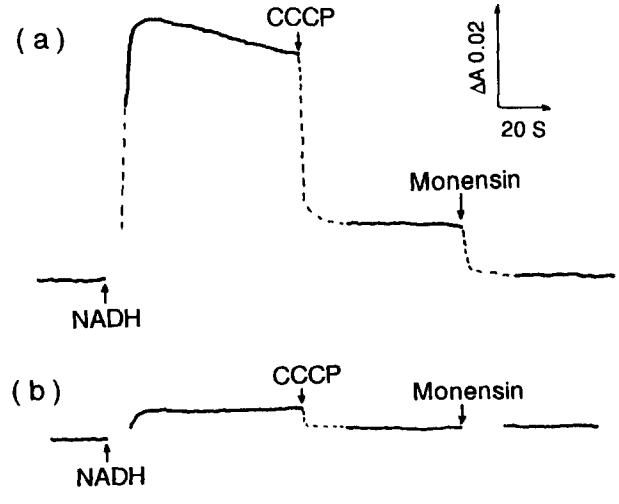

Fig. 3. Generation of a membrane potential and its sensitivity to CCCP (3 μM) and monensin (5 μM) in the inverted membrane vesicles from *H. influenzae*. The reaction was carried out in the NaCl medium (a) or in the KCl medium (b) as described in section 2.4, and each reagent was added at the arrows.

These results are in agreement with functioning of a respiration-driven Na^+ pump.

The effects of CCCP on the growth of *H. influenzae* were also examined. The growth at pH 6.5 and 7.5 was completely stopped by 10 μM CCCP, whereas the growth at pH 8.5 was not affected even in the presence of 30 μM CCCP. The CCCP-resistant growth at alkaline pH was the same as observed with the marine *V. alginolyticus* [4].

3.4. Conclusion

We found a gene cluster in the genome of *H. influenzae*, that is almost identical to the *nqr* operon encoding the Na^+-translocating NADH-quinone reductase from the marine *V. alginolyticus*. The respiratory chain of *H. influenzae* was examined to confirm the expression of a *nqr* operon. All the results obtained in this paper supported the presence of Na^+-translocating NQR in the membranes of *H. influenzae*. This is the first report to show the presence of redox-coupled Na^+ pump in *H. influenzae*. *H. influenzae* does not belong to marine and moderately halophilic bacteria, but some strains of Haemophilus species are known to require 1.0–1.5% (w/v) NaCl for optimum growth [14]. These results suggest that *H. influenzae* has some genetic relations with marine bacteria.

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