Properties of the Membrane-Bound NADH: Menadione Oxidoreductase in the Aerobic Respiratory Chain of *Bacillus cereus*

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Membranes prepared from *Bacillus cereus* KCTC 3674, grown aerobically on a complex medium, oxidized NADH exclusively, whereas deamino-NADH was little oxidized. The respiratory chain-linked NADH oxidase system exhibited an apparent K_m value of approximately 65 μM for NADH. On the other hand, the enzymatic properties of the NADH: menadione oxidoreductase of NADH oxidase system were examined. The maximum activity of NADH: menadione oxidoreductase was obtained at pH 9.5 in the presence of 0.1 M KCl (or NaCl). The NADH: menadione oxidoreductase activity was very resistant to the respiratory chain inhibitors such as rotenone, capsaicin, and $AgNO_3$. Interestingly, the activity was stimulated by the 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO).

Key words : *Bacillus cereus* KCTC 3674, aerobic respiratory chain, NADH: menadione oxidoreductase

Introduction

Although the NADH dehydrogenases (NDH) in the bacterial respiratory chains are referred to as NADH:ubiquinone oxidoreductase, many bacteria have been known to possess quinones other than ubiquinone [2]. Thus, the name NADH: quinone oxidoreductase in bacteria seems more appropriate than NADH: ubiquinone oxidoreductase.

Three types of NADH: quinone oxidoreductases in the respiratory chain of bacteria have been reported [12]. They are the H^+-translocating NADH: quinone oxidoreductase (designated NDH-1), the Na^+-translocating NADH: quinone oxidoreductase (designated Na^+-NDH), and the NADH: quinone oxidoreductase lacking an energy coupling site (designated NDH-2). In general, NDH-1 or Na^+-NDH reacts with deamino-NADH as well as with NADH, shows high affinities for NADH, and possesses an energy coupling site [6,8]. In contrast, NDH-2 reacts very little with deamino-NADH, but with NADH without any energy coupling site, and shows low affinities for NADH [6-8]. *Escherichia coli* [7], *Vibrio alginolyticus* [9,10], and *Thermus thermophilus* HB-8 [13] are known to possess two different types of NADH: quinone oxidoreductases. *Zymomonas mobilis* is known to possess only NADH: quinone oxidoreductase lacking the energy coupling site [7].

Respiratory chain inhibitors have proved to be a useful tool for probing the mechanism of electron transfer and proton or sodium translocation in the respiratory chain. Generally, NDH-1 is inhibited by the respiratory inhibitors rotenone and capsaicin, whereas NDH-2 is only slightly inhibited by these inhibitors [11,12].

Interestingly, Na^+-NDH is known to be very resistant to rotenone and capsaicin [12], but highly sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide [10] and $AgNO_3$ [1].

Takao Yagi showed that the respiratory inhibitor capsaicin inhibited H^+-translocating NADH: quinone oxidoreductases, and did not inhibit the NADH oxidase and NADH: quinone oxidoreductase of *Bacillus subtilis* [11]. To date, little detailed study has been done on the enzymatic and energetic properties of the aerobic respiratory chain-linked NADH oxidase system in the genus *Bacillus*. *Bacillus cereus* KCTC3674, which is a gram-positive facultative anaerobic spore-forming rod-shaped bacterium, is known to possess a menaquinone with seven isoprene units as the respiratory quinone [4]. I investigated the enzymatic properties of the NADH: menadione oxidoreductase on the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674, and report the results here.

Materials and methods

Bacterial strain and conditions

The bacterial strain used in this work was *B. cereus* KCTC 3674 [3-5]. The bacterium was grown aerobically at 37°C in a liquid medium which contained 0.5% poly-

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peptone and 0.5% yeast extract in 50 mM Tris-HCl buffer (pH 7.5). Preculture grown overnight was used to inoculate the main culture to give a turbidity of approximately 0.03.

Preparation of membrane vesicles for the determination of respiratory activities

For the preparation of membrane vesicles from *B. cereus* KCTC 3674, the protoplast formation was carried out at 37°C. Cells harvested in logarithmic growth phase were suspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA at a concentration of 1 g (wet weight) per 80 ml. Lysozyme was added at a concentration of 300 μg/ml (freshly prepared). The mixture was incubated for 30 min, after which the protoplasts were harvested by centrifugation at 14,000× *g* for 30 min. Protoplasts were washed once in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA, and centrifuged at 14,000× *g* for 30 min at 4°C. Washed protoplasts were resuspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA to give a concentration of 20 ml/g, and protoplast suspensions were passed through a French pressure cell twice at 25,000 psi. Unbroken cells and cell debris were removed by centrifugation at 10,000× *g* for 10 min at 4°C, and the supernatant was centrifuged at 120,000× *g* for 2 hr at 4°C to sediment the membrane fraction. A membrane pellet was washed in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA. After sedimentation at 120,000× *g* for 2 hr at 4°C, inverted membrane vesicles were rewashed in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol. Inverted membrane vesicles were resuspened in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol at a protein concentration of about 25 mg/ml, and stored at -80°C.

Measurement of enzyme activities

The the NADH oxidase activity was measured at 37°C from a decrease in A_{340} by using varian Cary 3E spectrophotometer. The assay mixture of NADH oxidase contained 125 μM NADH or deamino-NADH in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 M KCl. The assay was started by addition of 200 μg of membrane protein. Activity was calculated by using a millimolar extinction coefficient of 6.22. The assay mixture for NADH: menadione oxidoreductase contained 200 μg of membrane protein, 30 mM KCN, and 150 μM menadione in 2 ml of 50

mM CAPSO-HCl (pH 9.5). The reaction of the NADH: menadione oxidoreductase was started by the addition of 125 μM NADH. The activity of NADH: menadione oxidoreductase was measured at 340 nm, and was calculated by using a millimolar extinction coefficient of 6.22.

Protein determination

Protein was measured by Bio-Rad protein assay, based on the method of Bradford, by using bovin serum albumin as a standard.

Results and Discussion

Enzymatic properties of the membrane-bound NADH oxidase

The effects of salts and pH on the NADH oxidase activity were examined with inverted membrane vesicles prepared from *B. cereus* KCTC 3674. As shown in Fig. 1, the membrane vesicles oxidized NADH (closed symbols), but very little deamino-NADH (open symbols) as a substrate. The rate of NADH oxidation was slightly activated by monovalent cations including Na^+ and K^+, but was little activated by Li^+ (data not shown). The maximum activity of NADH oxidase was obtained in the presence of 0.1 M KCl or NaCl. The optimal pH for NADH oxidation in the presence of 0.1 M KCl was 8.5 (data not shown). The
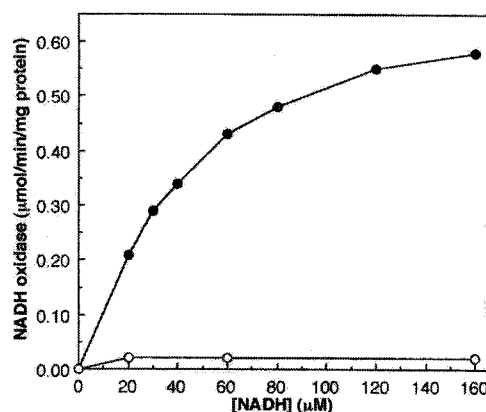

Fig. 1. Ability of NADH oxidase to oxidize NADH or deamino-NADH. The activities of NADH and deamino-NADH oxidases were measured in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 M KCl and different concentrations of NADH (closed symbols) or deamino-NADH (open symbols). The assay was started by addition of membrane suspensions containing about 200 μg of protein at 37°C.

NADH oxidase exhibited an apparent K_m value of approximately 65 μM for NADH. These results indicate that the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674 is the enzymatic system lacking an energy coupling site.

The effects of salts, pH, and respiratory inhibitors on of the NADH:menadione oxidoreductase activity

The NADH: menadione oxidoreductase activity was very slightly stimulated by Na^+ and K^+ at a concentration of 0.1 M (Fig. 2A). Its optimal pH was 9.5 (Fig. 2B). Interestingly, the respiratory inhibitor HQNO, which acts at the b cytochromes and also inhibits the Na^+-translocating NADH: quinone oxidoreductase, stimulated the NADH: menadione oxidoreductase activity (Fig. 3A). However, the NADH: menadione oxidoreductase were very resistant to the respiratory chain inhibitors such as rotenone and capsaicin, which inhibit the energy-transducing NADH: quinone oxidoreductase (Fig. 3B and C).

Effect of Ag^+ on the enzyme activities of the NADH oxidase system

AgNO3 is known to inhibit Na^+-translocating NADH: quinone oxidoreductase [1]. As shown in Fig. 4, the membrane-bound NADH oxidase activity of *Bacillus cereus* KCTC 3674 was highly sensitive to Ag^+ (closed squares). In

Fig. 3. Effects of respiratory chain inhibitors on the NADH: menadione oxidoreductase. The NADH: menadione oxidoreductase activity was measured with various concentrations of HQNO (A), rotenone (B), and capsaicin (C). The assay mixture of NADH: menadione oxidoreductase contained 200 μg of membrane protein, 30 mM KCN, 150 μM menadione in 2 ml of 50 mM CAPSO-HCl (pH 9.5) containing 0.1 M NaCl. All assays were started by addition of 125 μM NADH.

Fig. 4. Effect of AgNO3 on the enzyme activities of NADH oxidase system. NADH oxidase (closed squares), NADH: ubiquinone-1 oxidoreductase (closed circles) and NADH: menadione oxidoreductase (open circles).

contrast, the activities of NADH: ubiquinone-1, and NADH: menadione oxidoreductases were not affected by Ag^+ at all. Thus, Ag^+ inhibits the quinol oxidase segment of *Bacillus cereus*3674, but not NADH: quinone oxidoreductase.

Acknowledgement

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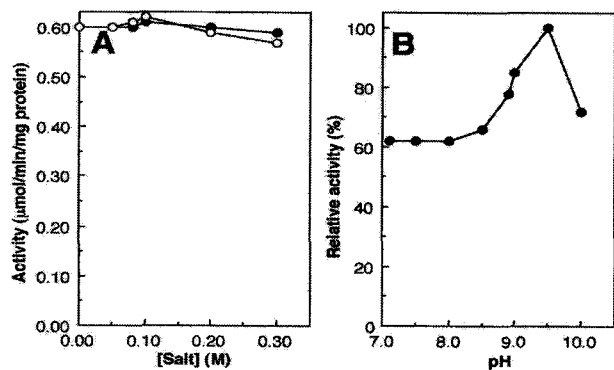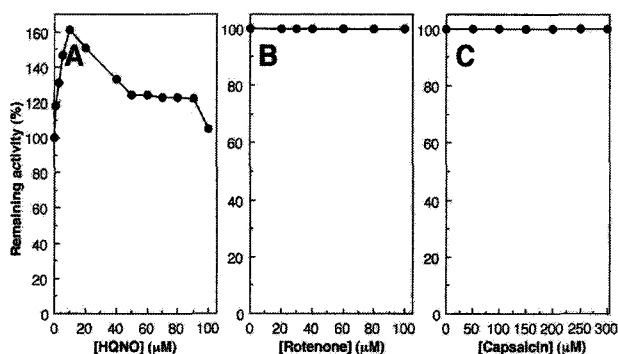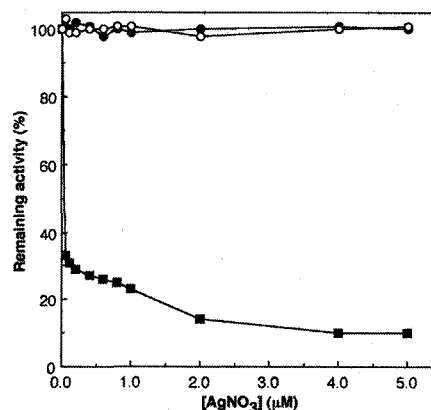1. Asano, M., M. Hayashi, T. Unemoto and H. Tokuda. 1985.

Fig. 2. Effects of salts and pH on the NADH: menadione oxidoreductase activity. (A) The assay mixture of NADH: menadione oxidoreductase (2 ml) contained 50 mM CAPSO-HCl (pH 9.5), 200 μg of membrane protein, 30 mM KCN, 150 μM menadione, and various concentrations of NaCl (closed circles), or KCl (open circles), (B) The NADH: menadione oxidoreductase activity was determined at various pHs. Buffers used at 50 mM was Tris-HCl (pH 7.1 to 8.9) and CAPSO-HCl (pH 9 to 10). All assays were started by addition of 125 μM NADH at 37°C.

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초록 : Bacillus cereus의 호기적 호흡쇄에 있어서 세포질막 내에 존재하는 NADH: menadione oxidoreductase의 특성

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호기적으로 자란 Bacillus cereus KCTC 3674로 부터 조제된 막은 NADH만을 산화하고, deamino-NADH는 거의 산화하지 않았다. 호흡쇄와 연계된 NADH oxidase계는 K_m 값이 약 65 μM이였다. 한편, NADH oxidase계 중 NADH: menadione oxidoreductase의 효소학적 특성이 조사되었다. NADH: menadione oxidoreductase의 최고활성은 0.1 M KCl (또는 NaCl) 존재 하에서 pH 9.5에서 얻어졌다. NADH: menadione oxidoreductase의 활성은 rotenone, capsaicin, AgNO₃와 같은 호흡저해제에 매우 저항적이였다. 그러나 매우 흥미롭게도 NADH: menadione oxidoreductase의 활성은 HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide)와 같은 저해제에 의해서는 오히려 촉진되어 졌다.