Journal of Biochemistry and Molecular Biology, Vol. 37, No. 6, November 2004, pp. 753-756

JOURNAL OF
Biochemistry and Molecular Biology

Short communication

Enzymatic Properties of the Membrane-bound NADH Oxidase System in the Aerobic Respiratory Chain of *Bacillus cereus*

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Received 8 July 2004, Accepted 27 October 2004

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 exhibited an apparent K_m value of approximately 65 μM for NADH. The maximum activity of the NADH oxidase was obtained at about pH 8.5 in the presence of 0.1 M KCl (or NaCl). Respiratory chain inhibitor 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) inhibited the activity of the NADH oxidase by about 90% at a concentration of 40 μM. Interestingly, rotenone and capsaicin inhibited the activity of the NADH oxidase by about 60% at a concentration of 40 μM and the activity was also highly sensitive to Ag^+.

Keywords: Aerobic respiratory chain-linked NADH oxidase system, *Bacillus cereus* KCTC 3674, Respiratory chain inhibitor

Introduction

Three types of NADH oxidase system in the respiratory chain of bacteria have been reported (Yagi *et al*., 1998). They are the H^+-translocating NADH oxidase system (designated NDH-1), the Na^+-translocating NADH oxidase system (designated Na^+-NDH), and the NADH oxdase system 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 (Matsushita *et al*., 1987; Kim *et al*., 1991). In contrast, NDH-2 reacts little with deamino-NADH, whereas reacts with NADH without any energy coupling site and shows low affinities for NADH (Matsushita *et al*., 1987; Kim *et al*., 1991; Kim *et al*., 1995). *Escherichia coli* (Matsushita *et al*., 1987), *Vibrio alginolyticus* (Tokuda, 1983; Tokuda and Unemoto, 1984), and *Thermus thermophilus* HB-8 (Yagi *et al*., 1988) 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 (Kim *et al*., 1995).

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 chain inhibitors rotenone and capsaicin, whereas NDH-2 is only slightly inhibited by these inhibitors (Yagi, 1990; Yagi *et al*., 1998). Interestingly, Na^+-NDH is known to be very resistant to rotenone and capsaicin (Yagi *et al*., 1998), but the NADH:quinone oxidoreductase of Na^+-NDH is highly sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (Tokuda and Unemoto, 1984) and $AgNO_3$ (Asano *et al*., 1985).

Takao Yagi showed that the respiratory chain inhibitor capsaicin inhibited H^+-translocating NADH:quinone oxidoreductases, and did not inhibit the NADH oxidase and NADH:quinone oxidoreductase of *Bacillus subtilis* (Yagi, 1990). However, to date, little detailed studies have been done on the enzymatic properties of the aerobic respiratory chain-linked NADH oxidase system in the genus *Bacillus. Bacillus cereus* KCTC 3674, 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 (Kim *et al*., 1998). In the present work, we investigate the enzymatic properties of the aerobic respiratory chain linked-NADH oxidase system of *B. cereus* KCTC 3674 and report the results here.

Materials and Methods

The bacterial strain used in this work was *B. cereus* KCTC 3674 (Kim *et al*., 1998; Kim *et al*., 2001). The bacterium was grown aerobically at 37°C in a liquid medium which contained 0.5% polypeptone and 0.5% yeast extract in 50 mM Tris-HCl buffer (pH 7.5). A preculture grown overnight was used to inoculate the main

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culture to give a turbidity of approximately 0.03.

For the preparation of membranes 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 h 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 h at 4°C, membranes were rewashed in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol. Membranes 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.

The activity of NADH oxidase 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 μM 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 oxidoreductases of NADH oxidase system contained 200 μg of membrane protein, 30 mM KCN, and a given electron acceptor in 2 ml of buffer. Ubiquinone-1 (Q-1) reductase activity was measured at 340 nm with 20 μM Q-1 and menadione reductase activity was measured at 340 nm with 150 μM menadione. Activity was calculated by using millimolar extinction coefficients of 6.81 and 6.22, respectively, for Q-1 and menadione. The reaction of oxidoreductase was started by addition of 125 M NADH.

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

The effects of salts and pH on the NADH oxidase activity were examined with membranes prepared from *B. cereus* KCTC 3674. As shown in Fig. 1, the membranes oxidized NADH (closed circles), but very little deamino-NADH (open circles) as a substrate. The respiratory chain-linked NADH oxidase exhibited an apparent K_m value of approximately 65 μM for NADH (data not shown). The rate of NADH oxidation was slightly activated by 0.1 M NaCl (closed circles) and KCl (open circles), but little by LiCl (closed squares) (Fig. 2A). The optimal pH for NADH oxidation in the presence of 0.1 M KCl (or 0.1 M NaCl) was 8.5 (Fig. 2B).

The activity of the NADH oxidase was inhibited completely

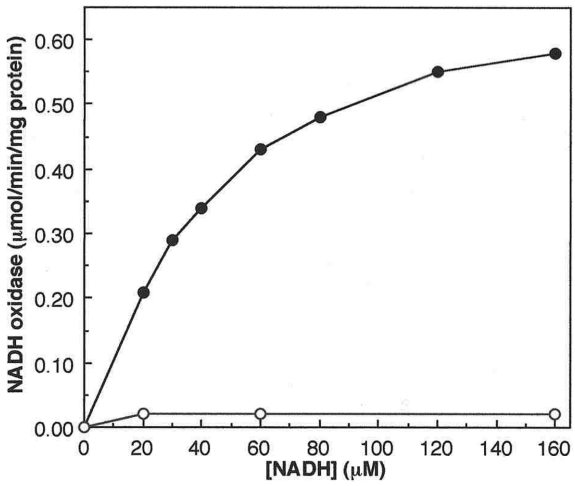

Fig. 1. Ability of the NADH oxidase to oxidize NADH or deamino-NADH. The activities of NADH and deamino-NADH oxidases were measured in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 μM KCl and different concentrations of NADH (closed symbols) or deamino-NADH (open symbols). The assay was started by addition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

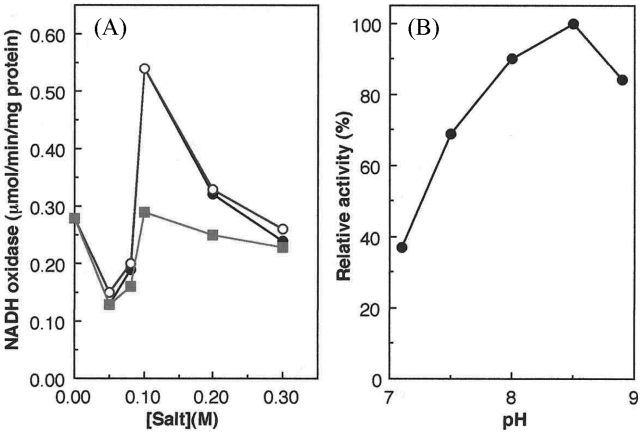

Fig. 2. Effects of salts and pH on the activity of the NADH oxidase. (A) The assay mixture of the NADH oxidase (2 ml) contained 50 mM Tris-HCl (pH 8.5), 125 μM NADH, and various concentrations of NaCl (closed circles), KCl (open circles), or LiCl (closed squares) (B) The activity of the NADH oxidase system was determined at various pHs in the presence of 0.1 μM KCl. Buffers used at 50 mM was Tris-HCl (pH 7.1 to 8.9). All assays were started by addition of membrane suspensions containing about 200 μg of protein at 37°C.

by 30 mM KCN (Fig. 3A). The respiratory chain inhibitor 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), which acts at the b cytochromes, inhibited the activity of the NADH oxidase by about 90% at a concentration of 40 μM (Fig. 3B). Rotenone and capsaicin, which inhibit the energy-transducing NADH:quinone oxidoreductase, also inhibited the activity by about 60% at a concentration of 40 μM (Fig. 3C and D).

$AgNO_3$ is known to inhibit the Na^+-translocating NADH:

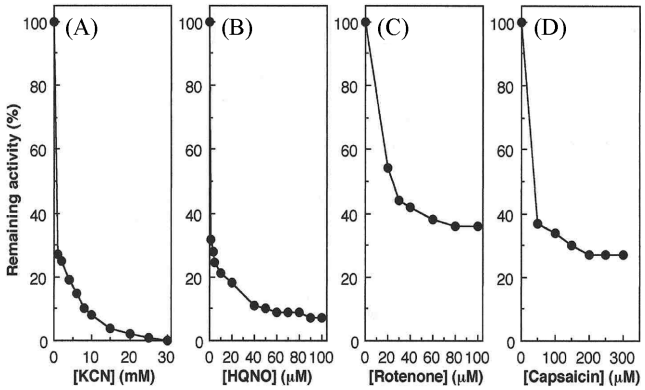

Fig. 3. Effects of respiratory chain inhibitors on the NADH oxidase. The activity of NADH oxidase was measured in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 μM KCl and various concentrations of KCN (A), HQNO (B), rotenone (C), and capsaicin (D). The assay was started by addition of membrane suspensions containing about 200 μg of protein at 37°C.

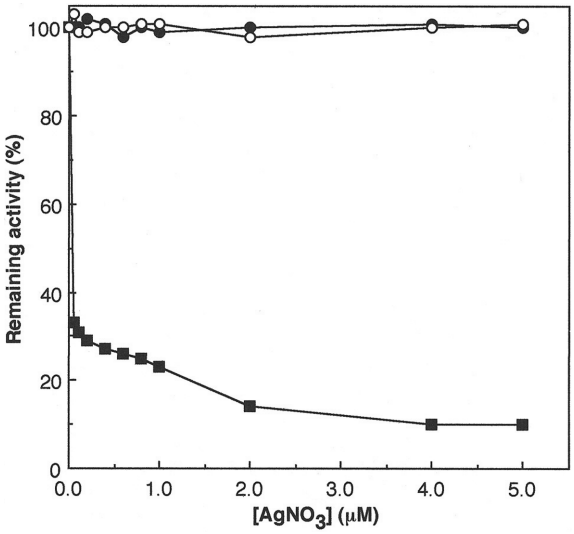

Fig. 4. Effect of $AgNO_3$ on the enzyme activities of the NADH oxidase. NADH oxidase (closed circles), NADH:ubiquinone1 oxidoreductase (closed squares), and NADH:menadione oxidoreductase (open circles).

quinone oxidoreductase (Asano *et al.*, 1985). As shown in Fig. 4, the membrane-bound NADH oxidase activity was highly sensitive to Ag^+ (closed squares). In contrast, the activities of NADH:ubiquinone-1 and NADH:menadione oxidoreductases were not affected by Ag^+ at all.

The results of this work clearly show that the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674 is quite different in enzymatic properties when compared to that of other bacteria. Membranes prepared from *B. cereus* KCTC 3674 oxidized NADH exclusively, whereas deamino-NADH was little oxidized. (Fig. 1), and exhibited an apparent K_m value of approximately 65 μM for NADH (data not shown). Generally, NADH oxidase system lacking an energy coupling site (NDH-2) oxidizes little deamino-NADH, and shows low affinities for NADH (Matsushita *et al.*, 1987; Kim *et al.*, 1991; Kim *et al.*, 1995). The activities of NADH oxidase and NADH:ubiquinone-1 oxidoreductase of *B. subtilis* membranes are little affected by capsaicin (Yagi, 1990). In contrast, capsaicin inhibited the activity of the NADH oxidase of *B. cereus* KCTC 3674 by about 60% at a concentration of 40 μM. The membrane-bound NADH oxidase activity was highly sensitive to Ag^+, whereas the activities of NADH:ubiquinone-1 and NADH:menadione oxidoreductases were not affected by Ag^+ at all, indicating that Ag^+ in *B. cereus* KCTC 3674 inhibited quinol oxidase segment exclusively.

B. cereus KCTC 3674 vigorously excretes proteases into the extracellular environment (Kim *et al.*, 2001). Thus, it is very difficult to prepare the functional inverted membrane vesicles for energy measurement experiment. Due to the lack of a functional *in vitro* system, little detailed study has been done on the energetic experiments in the genus *Bacillus*. There are the possibility that the NADH:menaquinone oxidoreductase of *B. cereus* KCTC 3674 may possess an energy coupling site by the results of respiratory chain inhibitors. Thus, we will try the preparation of inverted membrane vesicles for a functional *in vitro* energy measurement system.

Acknowledgments This work was financially supported by Changwon National University in 2003.

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