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A Novel Aerobic Respiratory Chain-Linked NADH Oxidase System in *Zymomonas mobilis*

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Membrane vesicles prepared from *Zymomonas mobilis* oxidized NADH exclusively, whereas deamino-NADH was little oxidized. In addition, the respiratory chain-linked NADH oxidase system exhibited only a single apparent K_m value of approximately 66 μM for NADH. The NADH oxidase was highly sensitive to the respiratory chain inhibitor 2-heptyl-4-hydroxyquinoline-*N*-oxide. However, the NADH:quinone oxidoreductase was not sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide and was highly resistant to another respiratory chain inhibitor, rotenone. Electron transfer from NADH to oxygen generated a proton electrochemical gradient (inside positive) in inside-out membrane vesicles. In contrast, electron transfer from NADH to ubiquinone-1 generated no electrochemical gradient. These findings indicate that *Z. mobilis* possesses only NADH:quinone oxidoreductase lacking the energy coupling site.

Zymomonas mobilis is well known as an obligately fermentative organism in which substrate-level phosphorylation from glycolysis provides the sole source of energy (9). In order to utilize glucose, fructose, and sucrose as carbon sources, this anaerobic bacterium uses the Entner-Doudoroff (4) pathway in conjunction with pyruvate decarboxylase and alcohol dehydrogenase. Although it is classified as an anaerobic bacterium, genus *Zymomonas* grows relatively well in the presence of oxygen (2, 3). Though the growth rate is lower in the presence of oxygen than under anaerobic conditions, the molar growth yield does not decrease (3). *Z. mobilis* appears to lack an oxidative electron transport phosphorylation (2). As a result, the taxonomic position of genus *Zymomonas* has not been fully established, mainly because of the uncertainties regarding the aerobic metabolism of these either aerotolerant or facultative anaerobes. So far, little information is available about the aerobic respiratory chain in *Z. mobilis*. It does appear to have cytochromes of the *b*, *c*, and *d* types (3, 10). According to a recent report of a study using electron transport inhibitors, *Z. mobilis* possesses, under aerobic conditions, a functional electron transport chain (12).

To date, no detailed study has been done on the enzymatic properties and bioenergetics of respiratory chain-linked NADH oxidase system in *Z. mobilis*. The present work describes the enzymatic properties of NADH oxidase and NADH:quinone oxidoreductase and the coupled bioenergetics of the aerobic respiratory chain in *Z. mobilis*. The results reported here also demonstrate the presence of only one type of NADH:quinone oxidoreductase (type 2 NADH:quinone oxidoreductase) in the aerobic respiratory chain-linked NADH oxidase system of *Z. mobilis.*

For the preparation of inside-out (ISO) membrane vesicles, *Z. mobilis* ZM1 was grown aerobically into mid-logarithmic phase at 30°C in liquid medium containing 1% yeast extract, 2% glucose, and 0.1% KH_2PO_4. ISO membrane vesicles were prepared as described previously (6), with the following modifications. Cells were harvested by sedimentation, washed twice in 50 mM Tris acetate (pH 6.5), and resedimented. Washed cells were resuspended in 50 mM Tris acetate (pH 6.5) containing 5 mM $MgSO_4$ at 6 ml/g (wet weight) of cells, and DNase I was added to 10 μg/ml. For the preparation of ISO membrane vesicles, the cell suspension was passed through the French pressure cell twice at 7,500 lb/in^2. Unbroken cells and cell debris were removed by sedimentation at 10,000 × *g* for 10 min, and a membrane pellet was obtained after sedimentation at 120,000 × *g* for 2 h. Membrane vesicles were resuspended in 50 mM Tris acetate (pH 6.5) containing 10% glycerol and kept frozen at −80°C.

For enzyme assays, NADH oxidase activity was measured at 30°C from a decrease in A_{340}. The assay mixture contained 0.22 mg of membrane protein in 2 ml of 50 mM Tris acetate (pH 6.5) containing 5 mM $MgSO_4$. Activity was calculated by using a millimolar extinction coefficient of 6.22. Each reductase activity of the NADH oxidase system was also measured spectrophotometrically at 30°C. The assay mixture for each reductase contained 0.22 mg of membrane protein, 20 mM potassium cyanide (KCN), and a given electron acceptor in 2 ml of 50 mM Tris acetate (pH 6.5) containing 5 mM $MgSO_4$. Ubiquinone-1 (Q1) reductase activity was measured at 340 nm with various concentrations of Q1, ferricyanide reductase activity was measured at 420 nm with 1 mM ferricyanide, and dichlorophenolindophenol (DCIP) reductase activity was measured at 600 nm with 50 μM DCIP. Activity was calculated by using millimolar extinction coefficients of 6.81, 1.0, and 16.5, respectively, for Q1, ferricyanide, and DCIP. All reactions were started by addition of 125 μM NADH or deamino-NADH (d-NADH).

Generation of $\Delta\Psi$ (inside positive) and ΔpH (inside acidic) in ISO membrane vesicles was monitored at 30°C by following fluorescence quenching of oxonol V and quinacrine, respectively, as described previously (5). The reaction mixture (2 ml, total volume) contained 50 mM Tris acetate (pH 6.5), 5 mM $MgSO_4$, 1 μM oxonol V or quinacrine, and 0.45 mg of membrane protein with or without 20 mM KCN. NADH and d-NADH were added at a final concentration of 0.2 mM. Q1, KCN, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP),

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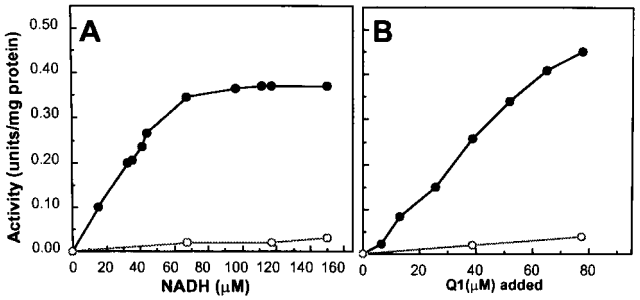

FIG. 1. Abilities of NADH oxidase and NADH:quinone oxidoreductase to oxidize NADH or d-NADH. (A) NADH oxidase (closed symbols) and d-NADH oxidase (open symbols) activities were measured with different concentrations of NADH and d-NADH. (B) NADH:quinone oxidoreductase activity was measured by dependency of NADH oxidation (closed symbols) or d-NADH oxidation (open symbols) in the presence of 20 mM KCN.

and $(NH_4)_2SO_4$ were added at final concentrations of 100 μM, 20 mM, 10 μM, and 100 mM, respectively.

Enzymatic properties of the aerobic respiratory chain-linked NADH oxidase system in *Z. mobilis*. In bacteria, a number of studies of the existence of two types of NADH:quinone oxidoreductase in the respiratory chain have been published (5, 8, 14, 16–18). In general, one of two types of NADH: quinone oxidoreductase (type 1) reacts with d-NADH as well as with NADH, shows high affinities for NADH (5 to 15 μM), possesses an energy coupling site, and is inhibited by the respiratory inhibitors rotenone and capsaicin. In contrast, the other (type 2) reacts exclusively with NADH without any energy coupling site, shows low affinities for NADH (50 to 60 μM), and is only slightly inhibited by rotenone or capsaicin (8, 15). Furthermore, Matsushita et al. (8) have observed two apparent K_m values for NADH in *Escherichia coli* membranes by assaying NADH oxidase and NADH:quinone oxidoreductase activities spectrophotometrically. While there have been numerous studies documenting the ability of *Z. mobilis* to produce ethanol efficiently under anaerobic conditions (1, 7, 9, 11, 13), only a few studies have investigated the aerobic metabolism of *Z. mobilis*. To elucidate the existence of the aerobic respiratory chain in *Z. mobilis*, ISO membrane vesicles were prepared from the cells grown aerobically. As shown in Fig. 1, membrane vesicles prepared from *Z. mobilis* oxidized only NADH, not deamino-NADH. Furthermore, membrane vesicles exhibited only a single apparent K_m value for NADH, the value of which is about 66 μM with oxygen or Q1 as an electron acceptor (data not shown). The activity of NADH:quinone oxidoreductase was dramatically increased with the amount of Q1 added in the presence of KCN (Fig. 1B). The activities of NADH oxidase and NADH:Q1 oxidoreductase in membrane vesicles reached a maximum at pH 6.5 (data not shown). Recently, Strohdeicher et al. (12) showed that the respiratory inhibitors rotenone and capsaicin caused only slight inhibition of NADH oxidase in cell extracts from *Z. mobilis*. On the other hand, the type 1 NADH:quinone oxidoreductase of the marine bacterium *Vibrio alginolyticus* shows slightly different properties. Type 1 NADH:quinone oxidoreductase of *V. alginolyticus* requires Na^+ for maximum respiratory activity and functions as an electrogenic Na^+ pump (5, 14). Moreover, the NADH: quinone oxidoreductase is resistant to rotenone but highly sensitive to a respiratory chain inhibitor, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). In the present investigation, the activity of NADH oxidase was completely inhibited by 20 mM KCN (Fig. 2A), which blocked the reduction of oxygen catalyzed by cytochrome aa_3 and was very sensitive to HQNO. In contrast, HQNO caused only slight inhibition of NADH:Q1 oxidoreductase activity in the same range of HQNO concentrations (Fig. 2B), and the NADH:Q1 oxidoreductase was highly resistant to the rotenone (Fig. 2C), which usually blocked type 1 NADH:quinone oxidoreductase, suggesting that electron flow from NADH to Q1 in the aerobic respiratory chain of *Z. mobilis* was not sensitive to HQNO and rotenone.

Generation of a proton electrochemical gradient in the NADH oxidase system of *Z. mobilis*. Generation of ΔΨ (inside positive) and ΔpH (inside acidic) in the NADH oxidase system was examined by measuring the fluorescence quenchings of oxonol V and quinacrine, respectively, with ISO membrane vesicles (5). Membrane vesicles prepared from *Z. mobilis* quenched the fluorescence of oxonol V upon the addition of NADH (Fig. 3A), indicating the generation of ΔΨ (inside positive). In contrast, the fluorescence quenching of oxonol V by the addition of d-NADH was little induced (data not shown). This membrane potential generated in the NADH oxidase system was completely collapsed by a proton conductor, CCCP (Fig. 3B), or a membrane permeable anion, SCN^-, which dissipates ΔΨ (data not shown). As shown in Fig. 3D, a large ΔpH was generated by the addition of NADH in the presence of SCN^-. The ΔpH generated by membrane vesicles was completely collapsed by $(NH_4)_2SO_4$ or KCN. However, no ΔpH was generated by the addition of d-NADH in the presence of SCN^- (Fig. 3F). On the other hand, electron flow from NADH to Q1 led to a very small transient fluorescence quenching of oxonol V or quinacrine in ISO membrane vesicles (Fig. 3C and E). However, these fluorescence quenchings were not collapsed by KSCN, $(NH_4)_2SO_4$, and CCCP, indicating that they were not true electrochemical potentials by NADH:quinone oxidoreductase segment (data not shown). As a consequence, the results obtained from ΔΨ and ΔpH sug-

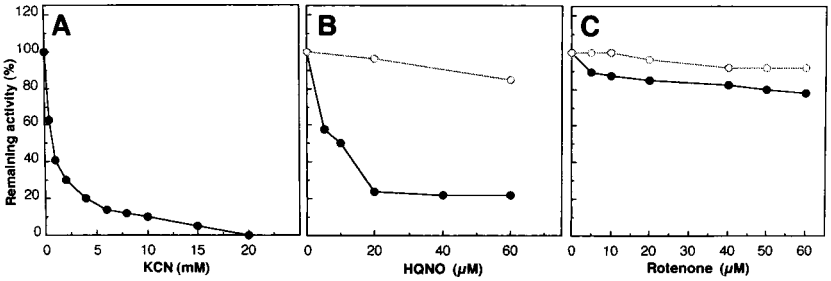

FIG. 2. Effects of electron transport chain inhibitors on the NADH oxidase and NADH:quinone oxidoreductase. (A) Activity of the NADH oxidase was measured with different concentrations of KCN. (B) Activities of the NADH oxidase and NADH:quinone oxidoreductase were measured with different concentrations of HQNO. (C) Activities of the NADH oxidase and NADH:quinone oxidoreductase were measured with different concentrations of rotenone. The activity of NADH:quinone oxidoreductase was measured in the presence of 20 mM KCN.

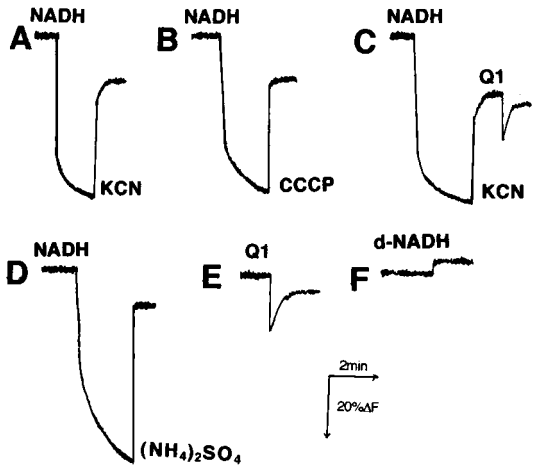

FIG. 3. Generation of $\Delta\Psi$ and ΔpH during electron transfer from NADH or d-NADH to Q1 or oxygen in ISO membrane vesicles. ISO membrane vesicles prepared from *Z. mobilis* were used to measure the generation of $\Delta\Psi$ (A to C) and ΔpH (D to F). Generation of ΔpH was measured in the presence of 10 mM KSCN. The reaction mixture in panel E contained 20 mM KCN and 0.2 mM NADH. NADH, d-NADH, KCN, CCCP, Q1, and $(NH_4)_2SO_4$ were added as specified in the text.

gested that electron flow from NADH to Q1 generated no electrochemical potential in ISO membrane vesicles from *Z. mobilis.*

On the basis of the results presented above, we conclude that *Z. mobilis* membranes contain only typical type 2 NADH: quinone oxidoreductase in the NADH oxidase and possess a bioenergetically functional aerobic respiratory chain. To determine whether its respiration is coupled to energy conservation, we present here several lines of evidence. (i) It appears to lack an oxidative electron transport phosphorylation (2), (ii) it produces a single net ATP molecule per molecule of glucose metabolized under both aerobic and anaerobic conditions (2), and (iii) the Pasteur effect is absent (2), which seems to be supported by the reports that $Y_{x/s}$ does not increase under aerobic conditions (3, 10). Moreover, our results suggest that NADH oxidation may not be an energy-yielding process because *Z. mobilis* membranes lack the type 1 NADH:quinone oxidoreductase. Also, the activity of succinate dehydrogenase on membrane vesicles prepared from the cells grown aerobically is not detected (data not shown). Therefore, the results presented above suggest that the respiration in *Z. mobilis* may not be coupled to energy conservation. Consequently, the sole ATP-producing reaction may be substrate-level phosphorylation in the anaerobic Entner-Doudoroff glucose catabolism pathway.

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