The Effect of Salts on Enzymes of the Respiratory Chain of Marine Bacterium Strain 1055-1

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The effect of salts on components of the respiratory chain of marine bacterium 1055-1 has been studied. Sodium ions activated the NADH oxidase system. This was not due to activation of NADH dehydrogenase but resulted from the activation of some other components that were membrane-bound. One of these activated components was involved in the 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO)-sensitive reduction of ubiquinone. In contrast, succinate oxidase was inhibited by high concentrations of salts and it was concluded that the inhibited component was succinate dehydrogenase.

INTRODUCTION

The uptake of amino acids such as L-alanine by cells and membrane vesicles of the marine Pseudomonas B-16 (Alteromonas haloplanktis) has been reported to be stimulated by Na+, and the energy for uptake supplied by respiration (MacLeod, 1968; Thompson & MacLeod, 1974; Hassan & MacLeod, 1975; Niven & MacLeod, 1978, 1980). In general, marine bacteria require Na+ and K+ for respiration with exogenous substrates. To understand the basis for these requirements, it is first necessary to study the effects of various salts on the respiratory chain in vitro and the permeation of substrates through the cell membrane. Reports of studies of respiration in Beneckea natriegens and the marine Pseudomonas BAL-31 made no mention of salt dependency (Franklin et al., 1971; Unemoto et al., 1977; Weston & Knowles, 1973).

The results presented in this paper suggest a role for Na+ in the respiration of cytoplasmic membrane fractions obtained from the Gram-negative marine bacterium strain 1055-1. The oxidation of NADH was activated by Na+.

METHODS

Strain and media. The organism used in this study is a Gram-negative marine bacterium strain 1055-1, first isolated from the North Pacific Ocean (Hidaka, 1965). Hidaka (1965) has studied the salt requirement for growth, the biochemical properties of the bacterium and its classification. Zobell 2216 E agar slants were used to maintain stock cultures. A modified Zobell medium 2216 E containing 0.5\% (w/v) polypeptone, 0.1\% (w/v) yeast extract and 0.01\% (w/v) ferric phosphate in Herbst sea water (pH 7.6) was used for the growth medium. Bacteria were inoculated into 100 ml medium in 500 ml flasks and cultivated at 30 °C with shaking at 120 rev. min⁻¹.

Preparation of membrane fractions. Bacteria were grown to the stationary phase at 30 °C, collected by centrifugation, and washed three times with 0.5 M-NaCl. The pellet was suspended in 0.5 M-sucrose and incubated at 30 °C with shaking to produce mureinoplasts (Costerton et al., 1967). These were collected by centrifugation at 20000 g for 15 min, and then washed with cold 0.5 M-sucrose. The mureinoplasts were suspended in a solution containing 0.5 M-sucrose, 2 mM-Tris/HCl (pH 7-6) and 100 µg lysozyme ml⁻¹, and incubated at 30 °C for 20 min; this resulted in the conversion of nearly all of the mureinoplasts to protoplasts. The protoplasts were collected by centrifugation at 20000 g for 10 min and suspended in 50 mM-Tris/HCl (pH 7-6) containing 5 mM-MgCl₂.
DNAase and RNAase were added to give 50 μg ml⁻¹ each (final concentration). The suspension was homogenized with a Teflon homogenizer, incubated at 30 °C for 15 min, and centrifuged at 75 000 g for 30 min. The pellet was washed once with 50 mM-Tris/HCl (pH 7-6), resuspended in 50 mM-Tris/HCl, and sonicated (20 kHz, 10–20 s) (Kaback, 1971).

Membranes were solubilized by adding Triton X-100 (0.5%, v/v) to the membrane suspension and the mixture was incubated for 30 min at 30 °C with shaking and then centrifuged for 1 h at 105 000 g. Between 30 and 40% of the membrane protein was found in the supernatant fraction.

Ubiquinone-depleted membranes were prepared by extracting lyophilized membranes (100 mg) with 10 ml n-pentane. The suspension was homogenized and centrifuged at 1500 g for 2 min. This procedure was repeated six times, and n-pentane remaining in the membranes was evaporated in vacuo to give the ubiquinone-depleted membranes (Ernst et al., 1959; Kroger et al., 1971).

Enzyme assays. NADH oxidase (EC 1.6.99.3): Activity was measured spectrophotometrically in an assay mixture containing 100 μM-NADH, 50 mM-Tris/HCl (pH 7-6) and enzyme. A millimolar absorption coefficient of \( \varepsilon_{260\text{nm}} = 6.2 \text{ M}^{-1} \text{cm}^{-1} \) was used for the calculation of activity. Alternatively, activity was determined by measuring the rate of utilization of dissolved oxygen in a medium containing 0-2 to 0.4 mM-NADH.

 Succinate oxidase (EC 1.3.99.1): Activity was determined by measuring the rate of utilization of dissolved oxygen in 50 mM-Tris/HCl (pH 7-6) solution containing 5 mM-ammonium succinate.

 Menadiol oxidase (EC 1.6.99.2): Reduced menadione was prepared by the method of Fieser (1940). The reaction was initiated by adding enzyme to a solution of 20 mM-imidazole/HCl (pH 6-3) containing 40 μM-menadion. Activity was calculated from the difference in absorbance at 262 nm between menadione and menadion (\( \varepsilon_{262\text{nm}} = 13.5 \text{ M}^{-1} \text{cm}^{-1} \)).

 Ascorbate:TMPD oxidase (EC 1.10.3.3): Activity was determined by measuring oxygen consumption polarographically (Lee et al., 1967). The enzyme solution was added to a mixture of 2 mM-ascorbate, 0.1 mM-N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and 50 mM-Tris/HCl (pH 7-6) and then incubated for 2 min.

 NADH dehydrogenase: Activity was measured spectrophotometrically using 2,6-dichlorophenolindophenol (DCIP), or ferricyanide or menadione as the electron acceptor. NADH:DCIP oxidoreductase activity was determined by measuring the reduction of DCIP associated with oxidation of NADH after adding enzyme to a reaction mixture containing 100 μM-NADH, 70 μM-DCIP, 1 mM-KCN and 50 mM-Tris/HCl (pH 7-6). An absorption coefficient of \( \varepsilon_{600\text{nm}} = 21 \text{ M}^{-1} \text{cm}^{-1} \) was used for the calculation of activity. NADH:ferricyanide oxidoreductase activity was determined by measuring the decrease at 420 nm due to the reduction of ferricyanide in an assay mixture containing enzyme, 100 μM-NADH, 750 μM-K₃Fe(CN)₆, 1 mM-KCN and 50 mM-Tris/HCl (pH 7-6). An absorption coefficient of \( \varepsilon_{420\text{nm}} = 1.0 \text{ M}^{-1} \text{cm}^{-1} \) was used for the calculation. NADH:menadione oxidoreductase activity was determined by measuring oxidation of NADH. The assay contained 100 μM-NADH, 200 μM-menadione, 1 mM-KCN and 50 mM-Tris/HCl (pH 7-6) and enzyme (Lanyi, 1969b). An absorption coefficient of \( \varepsilon_{260\text{nm}} = 6.2 \text{ M}^{-1} \text{cm}^{-1} \) was used for the calculation.

 Succinate dehydrogenase: Ferricyanide or phenazine methosulphate (PMS) was used as the electron acceptor. Succinate: PMS oxidoreductase activity was determined by measuring the absorbance of DCIP at 600 nm in a medium containing 10 mM-succinate, 70 μM-DCIP, 700 μM-PMS, 1 mM-KCN, 50 mM-Tris/HCl (pH 7-6) and enzyme. An absorption coefficient of \( \varepsilon_{600\text{nm}} = 21 \text{ M}^{-1} \text{cm}^{-1} \) was used for the calculation. Succinate:ferricyanide oxidoreductase was measured by the same method as NADH:ferricyanide oxidoreductase except that 10 mM-succinate was used instead of NADH.

 NADH:ubiquinone oxidoreductase: Activity was determined by measuring the oxidation of NADH by the enzyme in a medium containing 100 μM-NADH, 100 μM-ubiquinone-1 and 50 mM-Tris/HCl (pH 7-6) (Singer, 1974). In this experiment ubiquinone-depleted membranes were used as the source of enzyme.

 Protein determination. Protein concentrations were determined by the Lowry method.

**RESULTS**

Effect of salts on the oxidation of NADH and succinate. Figure 1 shows the effect of various chlorides on the activities of the NADH or succinate oxidases of the membranes. NADH oxidase activity was very low in the absence of added monovalent cations, but increased with increasing concentration of Na⁺. K⁺ and Li⁺ showed similar effects, but in each case the increase in activity was only half that obtained with Na⁺. In contrast, succinate oxidase showed maximum activity in the absence of added salts. The data in Table 1 show that monovalent cations have different effects on the \( K_m \) value of NADH oxidase.

Effect of salts on NADH dehydrogenase. As shown in Fig. 2, the activity of NADH dehydrogenase increased with increasing concentrations of Na⁺, K⁺ or Li⁺, reaching 160 to
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Fig. 1. Effect of salts on the oxidase activity of isolated membranes. NADH oxidase activity [measured spectrophotometrically and expressed as nmol NADH min\(^{-1}\) (mg protein\(^{-1}\))] and succinate oxidase activity [measured polarographically and expressed as nmol O\(_2\) min\(^{-1}\) (mg protein\(^{-1}\))] were measured in the presence of NaCl (●), KCl (Δ), LiCl (O) and MgCl\(_2\) (▲).

Table 1. \(K_m\) values of NADH oxidases for NADH in the presence of various salts

<table>
<thead>
<tr>
<th>Salt</th>
<th>Conc (mM)</th>
<th>(K_m) value (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>250</td>
<td>20</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>LiCl</td>
<td>250</td>
<td>9</td>
</tr>
<tr>
<td>KCl</td>
<td>250</td>
<td>50</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of salts on the NADH dehydrogenase activity of isolated membranes. NADH:DCIP oxidoreductase activity [expressed as μmol DCIP min\(^{-1}\) (mg protein\(^{-1}\))] and NADH:ferricyanide activity [expressed as μmol Fe(CN)\(_6^{3-}\) min\(^{-1}\) (mg protein\(^{-1}\))] were measured in the presence of NaCl (●), KCl (Δ), LiCl (O) and MgCl\(_2\) (▲).
Fig. 3. Effect of salts on the succinate dehydrogenase activity of isolated membranes. Succinate: PMS oxidoreductase activity [expressed as nmol DCIP min⁻¹ (mg protein)⁻¹] and succinate:ferricyanide oxidoreductase activity [expressed as μmol Fe(CN)⁶⁻ min⁻¹ (mg protein)⁻¹] were measured in the presence of NaCl (●), KCl (∆), LiCl (Ο) and MgCl₂ (▲).

Table 2. Effect of brief sonication on oxidase and dehydrogenase activities

Membranes in 50 mM-Tris/HCl (pH 7.6) with or without 0.25 mM-NaCl were sonicated for 15 s at 0 °C, or left untreated, and then assayed for oxidase and dehydrogenase activities.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Membranes</th>
<th>Oxidase activity [nmol O₂ min⁻¹ (mg protein)⁻¹]</th>
<th>Dehydrogenase activity [nmol Fe(CN)⁶⁻ min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>Native</td>
<td>Tris 6.3</td>
<td>Tris 281</td>
</tr>
<tr>
<td></td>
<td>Native</td>
<td>Tris + Na⁺ 69.9</td>
<td>Tris + Na⁺ 1574</td>
</tr>
<tr>
<td>Succinate</td>
<td>Native</td>
<td>Tris 32.5</td>
<td>Tris 335</td>
</tr>
<tr>
<td></td>
<td>Sonicated</td>
<td>Tris 25.0</td>
<td>Tris 281</td>
</tr>
<tr>
<td>Succinate</td>
<td>Sonicated</td>
<td>Tris 35.8</td>
<td>Tris 362</td>
</tr>
</tbody>
</table>

180% of the activity observed in the absence of added salts. Mg²⁺ (20 to 30 mM) also activated the enzyme. The effect of salts on the activity of solubilized NADH dehydrogenase (NADH:DCIP oxidoreductase) was similar to that for membranes, whereas when ferricyanide was used as the electron acceptor, monovalent cations did not activate the enzyme.

Effect of salts on succinate dehydrogenase. As shown in Fig. 3, when PMS was used as the electron acceptor each salt inhibited the enzyme. When ferricyanide was used, 50 mM K⁺, Na⁺ or Li⁺ activated the enzyme slightly and 10 mM-Mg²⁺ strongly activated the enzyme. At 0.5 M each cation was inhibitory.

Effect of sonication on oxidase and dehydrogenase activities. The data reported above were obtained using membranes sonicated for a short period. Sonication caused a threefold increase in the activity of NADH dehydrogenase in Tris/HCl and a fivefold activation in the presence of 0.25 mM-NaCl (Table 2). In contrast, NADH oxidase activity in 0.25 mM-NaCl increased twofold with sonication but did not increase in Tris/HCl. Succinate oxidase and succinate dehydrogenase activities were unchanged after sonication (Table 2).

Effect of salts on the activity of ascorbate:TMPD oxidase and menadiol oxidase. Ascorbate:TMPD oxidase activity of membranes increased by 30% in the presence of
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0.25 mM Na\(^+\) and by 20% in the presence of 0.25 mM K\(^+\) or Li\(^+\) compared with the activity in the absence of metal ion, but 50 mM Mg\(^{2+}\) caused only a 10% increase in activity. For oxidation of menadiol, the activity was doubled in the presence of 0.25 mM Na\(^+\), K\(^+\) or Li\(^+\).

**Effect of salt on the reduction of ubiquinone in ubiquinone-depleted membranes.** Usually, the reduction of ubiquinone by NADH is measured in the presence of KCN, but membranes of strain 1055-1 contain a cyanide-insensitive terminal oxidase (data not shown). Therefore, the reduction of ubiquinone was measured with ubiquinone-depleted membranes and exogenous ubiquinone. The reduction of ubiquinone-1 by NADH was rapid in medium without added salts. Li\(^+\) and K\(^+\), each at 0.25 mM, activated the enzyme by 14% and 4%, respectively, but 50 mM Mg\(^{2+}\) had no effect. 2-Heptyl-4-hydroxyquinoline N-oxide (HOQNO) at 10 \(\mu\)M slightly inhibited the activity in the presence of K\(^+\), Li\(^+\) or Mg\(^{2+}\), but the enzyme was more sensitive to HOQNO in the presence of Na\(^+\) than in its absence.

**Effect of Li\(^+\) on cyanide-sensitive terminal oxidase.** The membranes of strain 1055-1 contain a cyanide-sensitive terminal oxidase. NADH oxidase activity was completely inhibited by 50 mM Li\(^+\) in the presence of 1 mM KCN. This suggests that NADH oxidation was via a cyanide-sensitive terminal oxidase in the presence of Li\(^+\). K\(^+\) and Mg\(^{2+}\) had no effect on this enzyme.

**DISCUSSION**

NADH oxidase activity in cell-free extracts of the extreme halophile *Halobacterium cutirubrum* was strongly activated by salts of metal ions including NaCl (Hochstein & Dalton, 1968; Lanyi, 1969a). NADH oxidase of marine bacterium strain 1055-1 was particularly strongly activated by NaCl (Fig. 1). In addition, NADH dehydrogenase was activated by low concentrations of MgCl\(_2\). This effect was different from that seen in *H. cutirubrum*. The requirement for Na\(^+\) for activation of NADH oxidase was not due to activation of the NADH dehydrogenase portion of the respiratory chain (Fig. 2). The apparent activation of NADH dehydrogenase by sonication was presumably due to eversion of the membranes. The fact that NADH oxidase in sonicated membranes was activated in the presence of NaCl might also be due to the production of more everted membranes. However, the results shown in Table 2 and Fig. 2, suggest that the Na\(^+\) requirement of NADH oxidase was due to stimulation of a component of the respiratory chain located between NADH dehydrogenase and ubiquinone. The lack of activation of succinate oxidase and dehydrogenase by sonication might be due to the permeability of the membrane to succinate. Succinate dehydrogenase was concluded to be the rate-limiting site and also the site of salt inhibition.

Since the reduction of endogenous ubiquinone was too rapid to be measured, the reduction of ubiquinone added as an artificial electron acceptor was measured. Its reduction was only slightly inhibited by HOQNO. Therefore, most of the ubiquinone was reduced by an HOQNO-insensitive NADH dehydrogenase and the rest by an HOQNO-sensitive component which was especially activated by Na\(^+\). Although K\(^+\) was required for growth and for L-alanine uptake by strain 1055-1 (data not shown), K\(^+\) had little effect on NADH oxidase activity in membranes. Oxygen consumption by intact cells, as measured with an oxygen electrode (Singer, 1974), required both Na\(^+\) and K\(^+\) for activation. Similar results were obtained with marine *Pseudomonas* B-16, that is, intact cells required both Na\(^+\) and K\(^+\) for the uptake of amino acids, while uptake of amino acids by membranes vesicles required only Na\(^+\) (Sprott & MacLeod, 1972).

Of particular interest is the finding that Na\(^+\) alone stimulated the electron transport chain in membranes from strain 1055-1. Bacteria usually contain a much lower concentration of Na\(^+\) than is found in the outside medium. The fact that K\(^+\) is taken up by active transport in intact cells has been shown in marine bacteria. So far, though, no one has reported the existence of a Na\(^+\)/K\(^+\) ATPase in bacteria. Whatever the mechanism for transport of Na\(^+\) and K\(^+\), further studies will be necessary to elucidate how these ion fluxes affect the respiratory chain.
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REFERENCES


