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A CYTOCHROME THAT CAN PUMP SODIUM ION

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Previous studies have shown that the bacterium, Vitreoscilla, generates a respiratory-driven $\Delta\psi_{Na+}$. Two major respiratory electron transport proteins, NADH dehydrogenase (NADH:Quinone oxidoreductase), and cytochrome o terminal oxidase are candidates for the electrogenic Na^+ pumping that mediates the $\Delta\psi_{Na+}$ formation. The NADH oxidase activity of the membranes was enhanced more by Na^+ than by Li^+. The NADH:Quinone oxidoreductase activity in the respiratory chain was enhanced by Na^+ and Li^+, whereas the quinol oxidase activity of cytochrome o was enhanced specifically by Na^+, and not by Li^+, K^+, or choline. Purified cytochrome o, reconstituted into Na^+-loaded liposomes in the right-side-out orientation, catalyzed a net Na^+ extrusion when energized with Q_1H_2[1]. In non-loaded inside-out proteoliposomes, this cytochrome catalyzed a net uptake of $^{22}Na^+$ when energized with ascorbate/TMPD. Both Na^+-pumping activities were inhibited by CN^-. These results are consistent with the Vitreoscilla cytochrome o being a redox-driven Na^+ pump.

In agreement with the basic tenets of the chemiosmotic hypothesis, bacterial respiratory chains usually couple the free energy of respiratory electron transport to pump H^+ and generate a $\Delta\mu_{H+}$ which drives endergonic transmembrane reactions (1-3). Cytochromes aa_3, and o terminal oxidases have been identified as H^+ pumps in several bacteria (4-8). NADH dehydrogenase appears to pump H^+ in mitochondria (9), but it is not known whether it plays a similar role in bacteria. Investigations within the last few years demonstrate that Na^+ can replace H^+ as a coupling cation in certain bacterial energy transductions (see 10,11 for reviews), the first of which was the membrane-bound Na^+-pumping decarboxylases of several anaerobic species (10). Among aerobic bacteria, Na^+-pumping respiratory chains have been identified in marine alkalotolerant species of the family, Vibrio (11-14), and the halophiles Ba_1, Alcaligenes and Alteromonas (15-16). Specifically, the NADH:quinone oxidoreductase of these bacteria has been identified as a respiratory-driven Na^+ pump, but the terminal oxidase of their respiratory chain remains a H^+ pump. Also, a Na^+-motive terminal oxidase has recently been reported in the marine alkalotolerant Bacillus BTU (17) and E. coli growing at alkaline pH (18), but the specific cytochromes involved were not identified in either case. We reported earlier that Vitreoscilla, which is a non-marine, mildly alkalotolerant strict aerobe generates a $\Delta\psi_{Na+}$ via a respiratory-driven primary Na+ pumping system (19). To identify the specific Na^+ pump(s) involved in the Na^+-coupled energy transduction, we investigated two major respiratory proteins of the bacterium, and report here that the activities of NADH dehydrogenase and cytochrome o terminal oxidase are Na^+ dependent, and that the purified cytochrome o reconstituted into liposomes pumps Na^+.

Abbreviations: Q_1H_2, Ubiquinol-1; TMPD, N,N,N',N'-Tetramethyl-1,4-phenylenediamine.

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METHODS AND MATERIALS

Preparation of Membranes and Purification of Cytochrome o. Vitreoscilla cells were grown and processed as previously described (19). The preparation of membranes and purification of cytochrome o were performed as described (20).

Assay for NADH Oxidase Activity. This activity was assayed by measuring O_2 uptake as membranes oxidized NADH. The total volume of the reaction medium was 3 ml and contained 10 mM Tris-HCl (pH 7.6), 1.32 mg of membrane proteins, varying concentrations of NaCl or other salts, and 1 mM NADH which was added last to start the reaction. O_2 uptake was monitored polarographically using a YSI model 53 oxygen meter thermostated at 25°C. Initial velocity of the reaction at each salt concentration was calculated and plotted against salt concentration.

Assay for NADH Dehydrogenase Activity. This assay measured the NADH dehydrogenase activity of membranes using NADH and menadione as electron donor and acceptor, respectively, while terminal respiration was blocked with cyanide. The assay medium, in a final volume of 2 ml, contained 22.5 mM Tris-HCl (pH 7.6), 10 mM KCN, 50 μg membrane proteins, varying concentrations of NaCl or other salts, and 150 μM menadione. After a three-minute incubation at 25°C, 150 μM NADH was added to start the reaction. The absorbance decrease at 340 nm was monitored with a Cary 210 spectrophotometer. Initial velocity of the reaction at each salt concentration was calculated and plotted against salt concentration.

Assay for Menadiol Oxidase Activity of Cytochrome o. The menadiol used in this assay was prepared by complete reduction of menadione with $LiBH_4$. The assay medium, with a final volume of 2.0 ml contained 22.5 mM Tris-HCl (pH 7.6), 30-330 μg protein (depending on whether membranes or purified cytochrome o was used), and varying concentrations of NaCl or other salts. Following a one-minute incubation at 25°C, 75 μM menadiol was added to start the reaction which was monitored either polarographically for O_2 uptake using a YSI model 53 oxygen meter, or spectrophotometrically for absorbance increase at 262 nm with a Cary 210 spectrophotometer. The O_2 uptake or absorbance increase of the reaction mixture in the absence of sample was similarly measured at every salt concentration and used to correct test data for autoxidation of menadiol. This assay mixture also contained the same concentration of Triton X-100 as the experimental reaction mixture (the solvent for purified cytochrome o contained 0.5% Triton X-100). Initial velocity of the reaction at each salt concentration was calculated and plotted against salt concentration.

Assay for TMPD Oxidase Activity of Cytochrome o. This assay was performed essentially as that described above for menadiol oxidase activity except that 100 μM TMPD plus 800 μM ascorbate substituted for menadiol. Sample concentration was 0.53 mg protein/ml for membranes or 12 μg protein/ml for the purified enzyme. The reaction was monitored polarographically for O_2 uptake.

Reconstitution of Cytochrome o for Na^+ Extrusion. The proteoliposomes used in the assays for Na^+ extrusion required a net inward-orientation of the cytochrome o molecules, and a method which was successful in a similar reconstitution of E. coli's cytochrome o (21) was modified and used. E. coli phospholipids (40 mg) in 1.5 ml of reconstitution buffer (25 mM Tris-HCl, pH 7.7 containing 1 mM NaCl) was sonicated to translucency at 80 W in 15 second intervals. After adding octyl glucoside (1.25%) and purified cytochrome o (122 μg in 0.1 ml) the solution was incubated at 0°C for 20 minutes with mild stirring, and then diluted rapidly into 32 ml of reconstitution buffer at 0°C. Following a further five-minute incubation, the liposomes were collected by centrifugation at 165,000 x g, 4°C, for 45 minutes and resuspended in 1.2 ml of the reconstitution buffer, frozen in liquid nitrogen, thawed at 25°C, and sonicated for 10 seconds before being used for the assay.

Reconstitution of Cytochrome o for Na^+ Uptake. The proteoliposomes used in the Na^+ uptake assays required a net outward orientation of the cytochrome o molecules and were prepared using a modification of the spontaneous incorporation method that was employed for a similar incorporation of cytochrome c oxidase (22). This procedure was similar to that described for Fig. 4 except that, (a) NaCl was omitted from the reconstitution buffer, and (b) the freeze-thaw-sonication step was omitted, the resuspended liposomes being used immediately.

Assay for Na^+ Extrusion. The assay was performed at 25°C in a vessel fitted with a Na^+-sensitive electrode (Orion Research model 84-11) and containing 25 mM Tris-HCl (pH 7.7) and Na^+-loaded proteoliposomes (49 μg protein) in a total volume of 2.5 ml. The mixture was stirred at 25°C until the electrode response had stabilized. Q_1H_2 (100 μM) was then added to start the reaction and the electrode response was recorded automatically at 3-minute intervals by a printer interphased with the electrode. The control assay contained 1 mM KCN which was added prior to stabilizing the electrode.

Assay for Na^+ Uptake. This was performed similar to that reported by Hilpert and Dimroth (23). The assay medium (total volume 1 ml) contained 25 mM Tris-HCl (pH 7.7), 1.5 mM $^{22}NaCl$ (specific activity 2555 CPM/μg Na^+), proteoliposomes (29 μg protein), and 800 μM ascorbate/100 μM TMPD. The ascorbate/TMPD was added last to start the reaction. At appropriate times, 100 μl aliquots were removed and immediately passed over Dowex 50W(X8)-Li^+ columns (2 cm x 0.5 cm) to separate free $^{22}Na^+$ from that accumulated by the proteoliposomes. The proteoliposomes were eluted by washing the columns with 0.6 ml of 10 mM lithium phosphate (pH 7.7) and the entrapped $^{22}Na^+$ was assayed by liquid scintillation counting.

RESULTS AND DISCUSSION

Since $\Delta\psi$ in Vitreoscilla is generated by a Na^+ pump which directly couples the free energy of respiratory electron transfer to Na^+ pumping (19), the Na^+ pump would most likely be one of the electron transfer proteins in the respiratory chain to provide the tight coupling normally required for the efficient conversion of energy across cell membranes (1-3,24). Accordingly, the respiratory chain of Vitreoscilla was screened for redox proteins which depended on Na^+ for optimal activity, and this was done by assaying the NADH oxidase activity in membrane fragments as a function of the concentrations of Na^+ and Li^+ (as control). A plot of the NADH oxidase activity versus the concentrations of Na^+ and Li^+ (Fig. 1) revealed that (a) Na^+ enhanced the electron transfer activity 69% compared to a 20% enhancement by Li^+, and (b) the enhancement profile for the Li^+ control had only one peak (at 20 mM), whereas, that for Na^+ appeared to have a shoulder at 20 mM and a distinct peak at 75 mM Na^+. To investigate which electron transfer protein was specifically stimulated by Na^+, the effects of these cations on the individual activities of NADH dehydrogenase and cytochrome o, the two major redox proteins in the respiratory chain, were determined.

NADH dehydrogenase activity in membrane fragments was assayed by allowing the membranes to oxidize NADH using menadione as the electron acceptor while terminal electron transport was blocked with 10 mM CN^-. This activity increased 760% with increasing concentrations of Na^+, and reached saturation at about 300 mM Na^+ (Figure 2). Li^+ also stimulated the activity at low concentrations, maximally (720%) at about 125 mM, but higher Li^+ concentrations were inhibitory (Fig. 2). The effects of K^+ and choline (not shown) were also determined and were similar to those of Li^+ except that the inhibition at higher concentrations was less drastic. The menadiol oxidase activity of both the membrane-bound and solubilized, purified cytochrome o were assayed spectrophotometrically, in the presence of increasing concentrations of Na^+. In each case, the increase in activity reached saturation at about 300 mM Na^+ and was 100% for the membrane-bound enzyme (Fig. 3A), and 83% for the purified enzyme (Figs. 3B). In contrast, increasing concentrations of Li^+ had little or no effect on the cytochrome o activity of either the membrane-bound

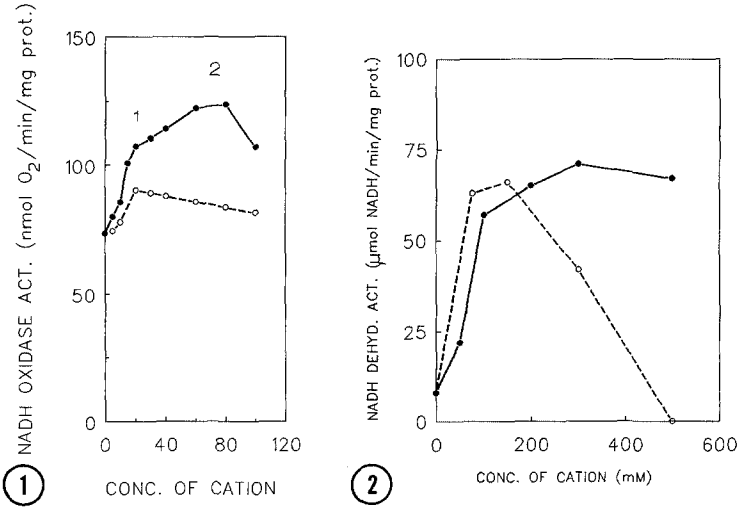


Fig. 1. Effects of Na^+ and Li^+ on the NADH oxidase activity of Vitreoscilla's respiratory chain. The NADH oxidase activity of membranes was assayed in the presence of varying concentrations of NaCl (●——●) or LiCl (o-----o) as described in Methods and Materials.

Fig. 2. Effects of Na^+ and Li^+ on membrane-bound NADH dehydrogenase activity. The assay measured the NADH dehydrogenase activity of membranes using NADH and menadione as electron donor and acceptor, respectively, while terminal respiration was blocked with CN^-. Details are given in Methods and Materials. (●——●), activity in the presence of NaCl; (o-----o), activity in the presence of LiCl.

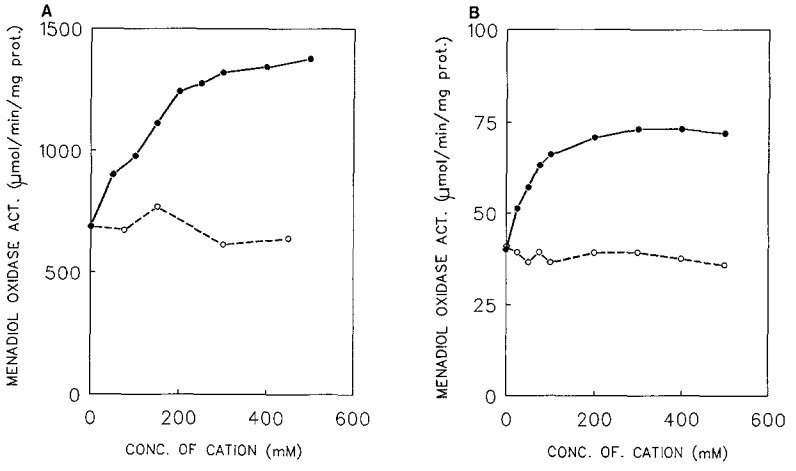
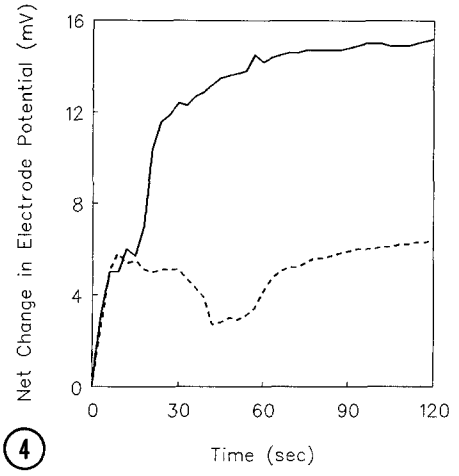


Fig. 3. (A) Effects of Na^+ and Li^+ on the menadiol oxidase activity of membrane-bound cytochrome o. The oxidation of menadiol by Vitreoscilla's membranes was assayed in the presence of varying concentrations of NaCl (●——●) or LiCl (o-----o) as described in Methods and Materials. (B) Effects of Na^+ and Li^+ on the menadiol oxidase activity of purified cytochrome o. The procedure was the same as in A except that purified cytochrome o replaced membranes. The specific activities in A and B were calculated based on initial velocities and have been corrected for the autoxidation of menadiol.

enzyme (Fig 3A), or the purified enzyme (Fig. 3B). When assayed polarographically, the results (not shown) were similar to those described above for the spectrophotometric assay (Fig. 3). The effects of K^+ and choline were also tested: They were similar to those of Li^+ in both assays. Also, when ascorbate/TMPD was substituted for menadiol as substrate, the results were also similar to those reported above: Na^+ increased the activity in a saturable manner, and the effect of Li^+ was negligible.

The enhancement of the quinol oxidase activity of cytochrome o by Na^+ was saturating and specific since neither Li^+, K^+, nor choline could substitute for Na^+. Therefore, the enhancement was not an ionic strength effect. Since the respiratory chain of Vitreoscilla has been demonstrated to generate a Na^+ gradient using primary Na^+ pumping, this effect of Na^+ on the oxidase activity of cytochrome o strongly implicated it as a primary Na^+ pump. Na^+ pumping by the Vitreoscilla cytochrome o was tested directly using two methods of proteolipososome reconstitution. The first was aimed at optimizing the extrusion of Na^+; in this method, cytochrome o was incorporated into preformed Na^+-loaded liposomes followed by the freeze-thaw-sonication steps which have been reported to favor a net inward orientation of reconstituted cytochrome o from E. coli (21). Energizing the proteoliposomes with Q_1H_2 drove a net extrusion of Na^+ (Fig. 4), and this transport was inhibited by CN^-. These results indicate a coupling of the Na^+ extrusion to the electron transfer reactions involving the Q_1H_2, cytochrome o, and oxygen. The second method was aimed at optimizing the accumulation of labelled Na^+. In this case, the proteoliposomes were prepared by a spontaneous incorporation method which has been shown to favor a net outward orientation of cytochrome c oxidase (22), and energizing was achieved with ascorbate/TMPD to enhance the sidedness of the reaction (12). The results (Fig. 5) show that in the presence of the ascorbate/TMPD, the liposomes rapidly accumulated the externally added $^{22}Na^+$, and this Na^+ transport was also inhibited by CN^-. When the proteoliposomes were tested in the absence of the ascorbate/TMPD, or when cytochrome o-free liposomes were tested in the presence of ascorbate/TMPD, the accumulation of $^{22}Na^+$ was practically nil (Fig. 5). The results from both types of proteoliposomes indicate a coupling between the Na^+ transport and the redox reactions involving the substrates, cytochrome o, and oxygen, and are strong evidence that cytochrome



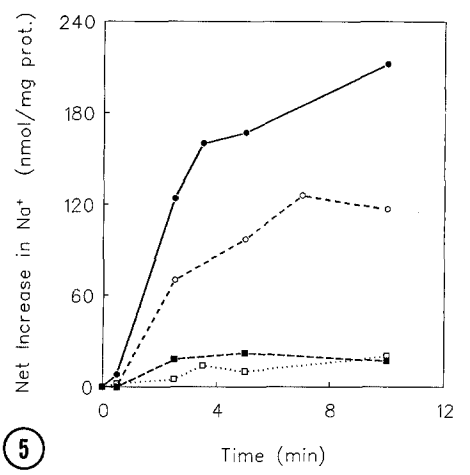


Fig 4. Na^+ extrusion by Na^+-loaded right-side-out cytochrome o proteoliposomes. The proteoliposomes were loaded with 1 mM Na^+ during preparation and assayed for extrusion of the Na^+ when energized with quinol-1, either in the absence (————) or presence (------) of 1 mM KCN. Details are given in Methods and Materials. The net change in the electrode potential is directly proportional to the net change in the concentration of Na^+ in the assay medium. The initial increase in electrode potential seen in both curves was due to the presence of Na^+ in the substrate solution because sodium borohydride was used to prepare the menadiol from menadione. The proteoliposomes were prepared as described in Methods and Materials.

Fig. 5. $^{22}Na^+$ uptake by inside-out cytochrome o proteoliposomes. The proteoliposomes were assayed for the uptake of $^{22}Na^+$ when energized with ascorbate/TMPD as described in Methods and Materials. The medium for each assay (total volume 1 ml) contained 25 mM Tris-HCl (pH 7.7), 1.5 mM $^{22}NaCl$ (specific activity 2555 CPM/μg Na^+), plus; proteoliposomes (29 μg protein) and 800 μM ascorbate/100 μM TMPD (●————●); or proteoliposomes, ascorbate/TMPD, and 1 mM KCN (○————○); or proteoliposomes minus ascorbate/TMPD (■– – –■); or liposomes (without cyt. o) plus ascorbate/TMPD (□····□). The proteoliposomes were prepared as described in Methods and Materials.

o pumps Na^+ coupled to the free energy of terminal oxidation. A preliminary estimate indicates a stoichiometry of 0.44 Na^+/O_2, which is low but reasonable, the low estimate probably resulting from significant error in O_2 uptake measurements caused by the high autoxidation rate (60%) of the TMPD.

The specificity of the enhancement of the cytochrome o oxidase activity by Na^+ was not observed for the NADH dehydrogenase activity of the membranes, which also showed some enhancement by lower concentrations of Li^+, K^+, and choline (Fig. 2). However, only Na^+ enhanced the activity in a saturable manner, suggesting a more specific ligand-receptor interaction between it and the enzyme, and raised the possibility that the enzyme may still be involved in respiratory-coupled Na^+ translocation. There are precedents for the involvement of NADH dehydrogenase in respiratory-driven Na^+ transport in prokaryotes: Vibrio alginoliticus, Vibrio costalica, and Ba_1, have been reported to employ their NADH:quinone oxidoreductase as a respiratory-driven Na^+ pump (13-16). Reconstitution experiments with the Vitreoscilla enzyme await its purification.

Additionally, the results in Figures 2 and 3 support the earlier observation on Fig. 1 that Na^+ enhanced terminal respiration with a profile which indicated two apparent peaks, which could mean that two components of the respiratory chain in the membranes were stimulated by Na^+, and one was also stimulated by Li^+. The activity of NADH dehydrogenase was enhanced by Na^+ and lower Li^+ concentrations (Fig. 2), characteristics which are similar to the enhancement peak at 20 mM Na^+ and Li^+ in Figure 1. Second, only Na^+ significantly enhanced the activity of cytochrome o (Figs. 3A and 3B), just as only Na^+ gave an enhancement peak around 75 mM in Figure 1. It is, therefore, reasonable to assign the first peak (shoulder) in Figure 1 to NADH dehydrogenase and the second peak to

cytochrome o. Although the cation concentrations at which the peak enhancement occurred in Figure 1 do not coincide with those in Figures 2 and 3, this is probably due to differences in the reaction systems utilized. The assays for Figure 1 were performed using a natural multicomponent redox system, i.e, the intact membrane, in which case, the activity of one redox enzyme at a particular Na^+ concentration is very likely affected by the other redox reactions. The assays described in Figures 2 and 3 were performed using single enzyme systems and the non-physiological electron carrier, menaquinone. For these reasons, significant differences in the reactivities of these enzymes may occur at the same salt concentration. Also, the menadiol oxidase activities of the membranes (Fig. 3A) were substantially higher than those of the solubilized, purified enzyme (Fig. 3B). One reason is that phospholipids, which are required for optimal activity of the purified cytochrome o (20), were omitted in the assay with the purified enzyme. In addition, the activities in Fig. 3A may have included contributions from cytochrome d which is present at a lower concentration than cytochrome o in the membrane of Vitreoscilla (25).

We conclude that cytochrome o from Vitreoscilla is a respiratory-driven Na^+ pump., and to the best of our knowledge, this is the first report of a cytochrome o that can function as a primary Na^+ pump.

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