Solubilization and reconstitution of the Na^+-motive NADH oxidase activity from the marine bacterium *Vibrio alginolyticus*

Hajime Tokuda

Research Institute for Chemobiodynamics, Chiba University, 1-8-1 Inohana, Chiba 280, Japan

Received 30 July 1984

The Na^+-motive NADH oxidase activity from *Vibrio alginolyticus* was extracted with octylglucoside and reconstituted into liposomes by dilution. On the addition of NADH, the reconstituted proteoliposomes generated $\Delta\psi$ (inside positive) and ΔpH (inside alkaline) in the presence of a proton conductor CCCP, and accumulated Na^+ in the presence of valinomycin. These results indicate that the NADH oxidase activity, reconstituted in opposite orientation, leads to the generation of an elctrochemical potential of Na^+ by the influx of Na^+.

NADH oxidase *Sodium pump* *Na^+ electrochemical potential* *Octylglucoside* *Reconstitution* *Marine bacterium*

1. INTRODUCTION

Vibrio alginolyticus possesses a respiration-coupled primary Na^+ pump [1,2]. The Na^+ pump generates the electrochemical potential of Na^+ which is resistant to CCCP. Examinations of respiratory activities in the Na^+ pump-defective mutants [3] revealed that the Na^+ pump was coupled to Na^+-dependent NADH oxidase which was highly sensiive to HQNO. NADH:quinone oxidoreductase was characterized as a Na^+-dependent, HQNO-sensitive site of NADH oxidase [4]. These results indicated that the translocation of Na^+ occurred at the step of quinol formation in the NADH oxidase. The reconstitution of the NADH oxidase activity was attempted and the generation of the electrochemical potential of Na^+ by the reconstituted proteoliposomes was examined.

Abbreviations: $\Delta\psi$, membrane potential; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; MET, 2-mercaptoethanol; octylglucoside, octyl-β-D-glucopyranoside; Q-1, ubiquinone-1

2. MATERIALS AND METHODS

2.1. *Solubilization and reconstitution*

Membranes were prepared from *V. alginolyticus* 138-2 grown on a medium as in [5]. Solubilization and reconstitution were performed based on [6]. The membranes (5 mg protein/ml) were resuspended in 0.1 M K-phosphate (pH 7.5), 0.5 mM EDTA-K, 2 mM MET, 5 mM Na_2SO_4. Liposomes prepared from acetone-washed soybean phospholipids (Sigma, Type 2-S) and octylglucoside were added at final concentrations of 20 mg/ml and 2%, respectively. The suspension (3 ml) was kept on ice for 20 min and centrifuged at 156500 × *g* for 1 h. The supernatant containing about 5 mg of solubilized membrane protein was mixed with 1.69 ml of liposomes (84.5 mg of lipid) and 188 μl of 20% octylglucoside. The mixture was kept on ice for 20 min and diluted into 134 ml of 0.1 M K-phosphate (pH 7.5), 0.5 mM EDTA-K, 2 mM MET, 5 mM Na_2SO_4. After 10 min incubation at room temperature, the resultant proteoliposomes were collected by centrifugation at 107000 × *g* for 1 h. The pellet was resuspended in

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

1 ml of 0.1 M K-phosphate (pH 7.5), 0.5 mM EDTA-K, 2 mM MET, 5 mM Na_2SO_4. The reconstituted proteoliposomes contained about 40 μg protein per mg phospholipid.

2.2. *Assays*

NADH oxidase and NADH:quinone oxidoreductase were spectrophotometrically assayed at 30°C as in [4]. $\Delta\psi$ (inside positive) and ΔpH (inside alkaline) were determined at room temperature by flow dialysis [1,7] from the distribution of $KS^{14}CN$ and [^{3}H]acetate (sodium salt), respectively. Accumulation of $^{22}Na^+$ was also determined at room temperature by flow dialysis. Protein was determined by the method in [8]. Internal space (1.3 μl/mg of phospholipid) of the reconstituted proteoliposomes was determined from their K^+-content as in [7].

3. RESULTS

3.1. *Respiratory activities*

NADH-dependent ubiquinol formation by the reconstituted proteoliposomes was examined in 0.1 M K-phosphate (pH 7.5), 0.5 mM EDTA-K, 2 mM MET, 10 mM KCN with or without 5 mM Na_2SO_4. In a typical experiment, specific activities of ubiquinol formation were 1.43 and 0.1 μmol/min per mg protein in the presence and absence of Na^+, respectively. Addition of a higher concentration of Na^+ gave little additional stimulation since the apparent K_m for Na^+ is about 6 mM in the presence of a high concentration of K^+ [5]. The Na^+-dependent ubiquinol formation was almost completely inhibited by 1 μM HQNO, a specific inhibitor of the Na^+ pump [4]. The proteoliposomes also demonstrated Na^+-dependent NADH oxidase activity which was sensitive to KCN and HQNO. However, the activity was substantially stimulated by the addition of Q-1, indicating a lack of endogenous quinone in the proteoliposomes. Therefore, generation of the Na^+ electrochemical potential by the proteoliposomes was determined in the presence of Q-1.

3.2. *Generation of the electrochemical potential of Na^+*

As shown in fig.1, the proteoliposomes accumulated $S^{14}CN^-$ on the addition of NADH and Q-1, indicating the generation of $\Delta\psi$ (61 mV, inside positive). In contrast, the proteoliposomes did not generate $\Delta\psi$ (inside negative) since no accumulation of tetraphenylphosphonium ion was detected. The $\Delta\psi$ was collapsed partially by CCCP and completely by valinomycin. The magnitude of $\Delta\psi$ in the presence of CCCP was 43 mV. When HQNO was added together with NADH and Q-1, generation of $\Delta\psi$ was significantly inhibited.

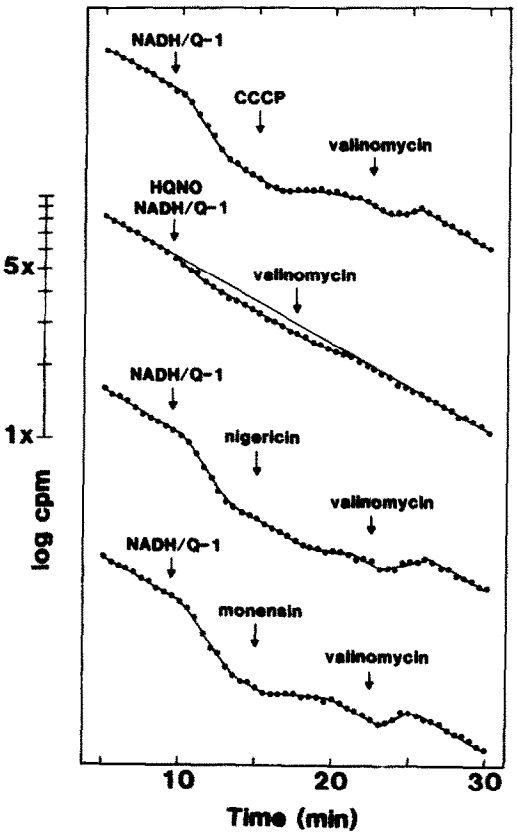

Fig.1. Generation of $\Delta\psi$ (inside positive) by the reconstituted proteoliposomes. $KS^{14}CN$ (59 μCi/μmol, 64 μM) was added at 0 time to 0.4 ml of the assay mixture containing 0.1 M K-phosphate (pH 7.5), 0.5 mM EDTA-K, 2 mM MET, 5 mM Na_2SO_4 and 1.1 mg protein/ml of the proteoliposomes. The buffer pumped through the lower chamber of the flow dialysis cell was the same as that in the upper chamber. Radioactivities in dialysate were continuously monitored as in [1]. Other additions: 9.8 mM NADH, 0.35 mM Q-1, 3 μM CCCP, 5 μM valinomycin, 2 μM HQNO, 1 μM nigericin and 25 μM monensin.

Although both nigericin and monensin are electroneutral ionophores, the generation of $\Delta\psi$ was slightly inhibited by these ionophores. Magnitudes of $\Delta\psi$ were 53 and 49 mV in the presence of nigericin and monensin, respectively.

On addition of NADH and Q-1, the proteoliposomes generated ΔpH (inside alkaline) which was monitored by the accumulation of [^{3}H]acetate (fig.2A). Note that the generation of ΔpH was stimulated when the proteoliposomes were treated with CCCP. Magnitudes of ΔpH were 34 and 24 mV in the presence and absence of CCCP, respectively. Moreover, the ΔpH was collapsed not only by nigericin but also by valinomycin. Similar results were obtained when 5,5′-dimethyloxazolidine-2,4-dione was used instead of acetate.

The proteoliposomes treated with valinomycin accumulated $^{22}Na^+$ on addition of NADH and Q-1 (fig.2B). Internal concentration of Na^+ at the steady-state level of accumulation was 3- to 4-fold higher than the external. CCCP did not inhibit the accumulation of Na^+ whereas monensin released accumulated Na^+. Note that the extent of Na^+ accumulation in the absence of valinomycin was considerably smaller than that in the presence of valinomycin.

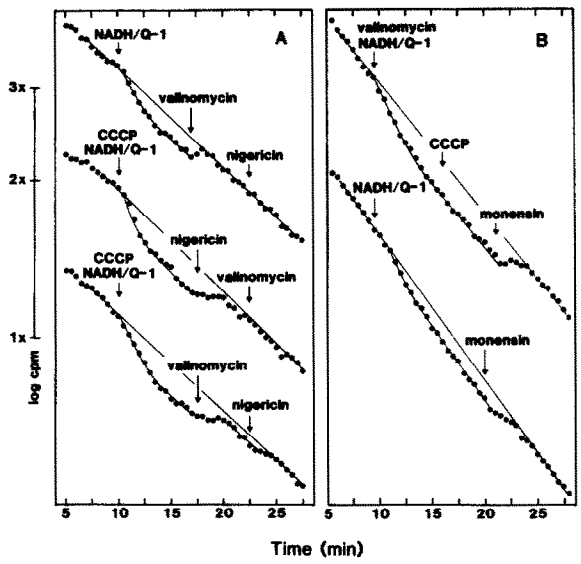

Fig.2. Generation of ΔpH (inside alkaline) and accumulation of Na^+ by the proteoliposomes. (A) ΔpH was determined from the distribution of [^{3}H]acetate (2 mCi/μmol, 20 μM) as in fig.1. (B) ^{22}NaCl (3.3×10^3 cpm/μl) was added at 0 time to 0.4 ml of the assay mixture containing the buffer given in fig.1 and 1.7 mg protein/ml of the proteoliposomes. The buffer pumped through the lower chamber contained K_2SO_4 instead of Na_2SO_4. Other additions were as in fig.1.

4. DISCUSSION

Since NADH is impermeable to the liposomal membrane, the NADH oxidase activity observed in the proteoliposomes seems to be derived from the NADH oxidase which is oriented opposite to that in whole cells. Indeed, the polarity of the electrochemical potential of Na^+ in the proteoliposomes was opposite to that in whole cells. The stimulation of Na^+ uptake into the proteoliposomes by valinomycin indicate that Na^+ influx is a primary electrogenic process. Since liposomes made from soybean phospholipids (Sigma, Type 2-S) are somewhat permeable to H^+ [9], the generation of inside-positive $\Delta\psi$ by the influx of Na^+ led to the extrusion of H^+ which resulted in the generation of inside-alkaline ΔpH, not only in the presence but also in the absence of CCCP. Therefore, collapse of $\Delta\psi$ by valinomycin concomitantly collapsed this ΔpH. Since the generation of the ΔpH restricts further extrusion of H^+, the collapse of the ΔpH by nigericin and, presumably, monensin may result in the diminution of $\Delta\psi$. In addition, since monensin converts Na^+ influx into H^+ influx, the magnitude of $\Delta\psi$ in the presence of monensin may be limited by H^+-permeability of the proteoliposomes. These seem to be the reasons for the partial collapse of $\Delta\psi$ by nigericin and monensin. Although it is not clear whether the proteoliposomes translocate H^+, the results here clearly indicate that the Na^+-dependent NADH oxidase of *V. alginolyticus* generates the electrochemical potential of Na^+ as a direct result of NADH oxidation.

ACKNOWLEDGEMENT

This work was supported by a grant from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

[1] Tokuda, H. and Unemoto, T. (1981) Biochem. Biophys. Res. Commun. 102, 265–271.
[2] Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014.
[3] Tokuda, H. (1983) Biochem. Biophys. Res. Commun. 114, 113–118.
[4] Tokuda, H. and Unemoto, T. (1984) J. Biol. Chem. 259, 7785–7790.
[5] Unemoto, T., Hayashi, M. and Hayashi, M. (1977) J. Biochem. (Tokyo) 82, 1389–1395.
[6] Newman, M.J. and Wilson, T.H. (1980) J. Biol. Chem. 255, 10583–10586.
[7] Tokuda, H., Nakamura, T. and Unemoto, T. (1981) Biochemistry 20, 4198–4203.
[8] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[9] Sone, N. and Yanagita, Y. (1984) J. Biol. Chem. 259, 1405–1408.