The Na^+/e^- stoichiometry of the Na^+-motive NADH : quinone oxidoreductase in *Vibrio alginolyticus*

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Abstract A method is proposed to estimate the stoichiometries of primary Na^+-pumps in intact bacterial cells. It is based on technique when the H^+/e^- stoichiometry is measured in the presence of protophorous uncoupler and in the absence of penetrating ions other than H^+. Under these conditions, the H^+ influx discharges membrane potential generated by the Na^+ pump so the Na^+/e^- and H^+/e^- ratios become equal. Using this approach it is shown that the Na^+/e^- ratio for the Na^+-motive NADH : quinone oxidoreductase of *Vibrio alginolyticus* is equal to 0.71 ± 0.06. The Na^+/e^- stoichiometry appears to be ≈ 1, provided that the contribution of the non-coupled NADH : quinone oxidoreductase, which is resistant to low HQNO concentrations, is taken into account.

Key words: Na^+/e^- stoichiometry; Na^+-motive NADH : quinone oxidoreductase; *Vibrio alginolyticus*

1. Introduction

The Na^+-motive NADH : quinone oxidoreductase is shown to operate in the initial span of the respiratory chain of the marine bacterium *Vibrio alginolyticus*. This electrogenic primary Na^+ pump generates a transmembrane difference in Na^+ electrochemical potentials ($\Delta\mu_{Na^+}$) [1,2]. The $\Delta\mu_{Na^+}$ formed is then utilised to support osmotic work, i.e. uphill transport of solutes [3], mechanic work, i.e. rotation of flagellum [4], and chemical work, i.e. oxidative phosphorylation [5–7]. Genes encoded for this enzyme were recently cloned and sequenced. It was found that the corresponding operon consists of six genes showing no substantial homology with other NADH : quinone oxidoreductases [8,9]. It was also established that the Na^+-NADH : quinone oxidoreductase contains FAD and a FeS cluster [9,10]. The functional mechanism of this enzyme still remains obscure. In particular, such a crucial parameter as the Na^+/e^- stoichiometry is unknown. In the series of experiments described below, we established this stoichiometry to be ≈ 1.

2. Materials and methods

2.1. Bacterial strains and growth conditions

V. alginolyticus, strain 138-2 (wild type), and a mutant strain, deficient in the Na^+-motive NADH : quinone oxidoreductase, Nap1 [2] were the generous gifts of Professor Tsutomu Unemoto. The cells were grown to the middle exponential phase at 37°C in medium containing 0.5 M NaCl, 10 mM KCl, 15 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 0.05% yeast extract, 0.4% (v/v) glycerol, 0.5 mM Na_2HPO_4, 50 mM Tris-HCl pH 8.6.

2.2. The stoichiometry measurement

The cells were harvested at $7000 \times g$ (10 min). Then, internal K^+ was replaced by Na^+ according to the modified method of Nakamura et al. [11]. The cells were suspended in a medium containing 0.4 M NaCl, 5 mM $MgSO_4$ and 50 mM diethylamine pH 8.8, incubated at room temperature for 10 min and sedimented at $7000 \times g$. This procedure was repeated twice. After this, the cells were washed twice with 0.2 M Na_2SO_4, 5 mM $MgSO_4$ and 20 mM HEPES-NaOH pH 7.5. Finally, the cells were washed with the incubation medium (0.2 M Na_2SO_4, 5mM $MgSO_4$, 10 mM glycerol, 0.1 mM HEPES-NaOH pH 7.5), re-suspended in this medium (to 1.5 mg cell protein $\times$ ml^{-1}) and injected into a 1.4-ml chamber equipped with a pH electrode. After saturating the mixture with argon, the chamber was closed and the pH level was adjusted to pH 7.5 with NaOH.

To measure the H^+/e^- stoichiometry, 5 nmol O_2 was added. The pH electrode was calibrated with 10 nmol additions from the argon-saturated H_2SO_4 solution. To estimate the H^+/e^- ratio, linear extrapolation was employed according to [12].

Respiration was measured by a Clark-type oxygen electrode.

Protein concentration was estimated by means of the biuret micromethod, using bovine serum albumin (Merck) as a standard.

3. Results and discussion

In 1982, Tokuda and Unemoto [1] showed that an O_2 addition to the *V. alginolyticus* cells in the presence of a protonophore gives rise to an alkalization of the medium. This effect was interpreted as the electrophoretic, protonophore-mediated H^+ uptake supported $\Delta\Psi$ which was generated by the Na^+-NADH : quinone oxidoreductase. Such an effect can be used to measure the Na^+/e^- stoichiometry of this enzyme. In fact, the number of H^+ ions imported by the cell in the presence of protonophore must be equal to the number of Na^+ ions exported by the Na^+-NADH : quinone oxidoreductase, provided that H^+ is the only ion discharging the Na^+ pump-generated $\Delta\Psi$. To minimize the contribution of other ion fluxes to the $\Delta\Psi$ discharge, we excluded K^+ and Cl^- from the incubation medium and replaced intercellular K^+ with Na^+.

As one can see in Fig. 1, the O_2 pulse in the presence of penetrating cation TPP^+ entails an H^+ efflux from the *V. alginolyticus* cells which is presumably a result of operation of the H^+-motive terminal oxidase [13]. On the other hand, the same O_2 pulse in the presence of protonophore CCCP gives rise to an opposite effect, i.e. an H^+ influx. This could be accounted for by an electrophoretic H^+ uptake caused by a discharge of the Na^+-NADH : quinone oxidoreductase-generated $\Delta\Psi$. No pH change occurs when both TPP^+ and CCCP

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Abbreviations: $\Delta\mu_{H^+}$ and $\Delta\mu_{Na^+}$, transmembrane differences in H^+ and Na^+ electrochemical potentials, respectively; $\Delta\Psi$, transmembrane difference in electric potentials; CCCP, m-chlorocarbonylcyanide phenylhydrazone; HQNO, 2-heptyl-4-hydrooxyquinoline N-oxide; TPP^+, tetraphenyl phosphonium; TTFB, 4,5,6,7-tetra-chloro-2-trifluoromethylbenzimidazole

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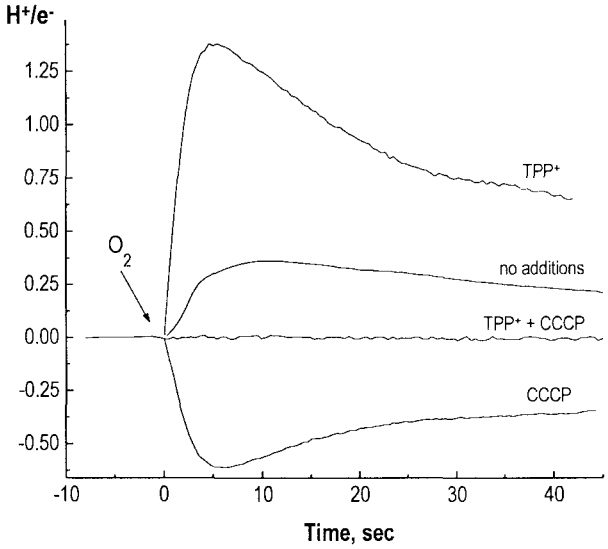


Fig. 1. The H^+/e^- stoichiometries in the *V. alginolyticus* 138-2 cells oxidizing glycerol. Additions: 5 mM TTP^+, 15 μM CCCP.

are added. In the absence of TPP^+ and CCCP, a very small H^+ efflux is observed.

The dependence of the H^+/e^- stoichiometry upon the CCCP concentration is shown in Fig. 2. According to these data, the maximal H^+/e^- ratio is reached at 15 μM CCCP. This ratio provided to be equal to 0.71 ± 0.06 (mean of 8 experiments). Similar H^+/e^- stoichiometry was achieved when using TTFB as a protonophorous uncoupler (data not shown).

No pH change during O_2 pulses in the presence of CCCP is observed in a mutant strain *V. alginolyticus* lacking Na^+-NADH : quinone oxidoreductase. On the other hand, the same mutant appears to be competent in the TPP^+-stimulated H^+ efflux when CCCP is absent (Fig. 3A). Magnitude of the O_2 pulse-induced H^+ influx into wild-type cells in samples with CCCP is strongly decreased when lactate substitutes for glycerol as the oxidation substrate (Fig. 3B). The same figure shows that the H^+ influx is arrested by very low HQNO concentrations. Parallel measurement of the respiration rate revealed that these HQNO concentrations lower the O_2 consumption by the *V. alginolyticus* cells by ≈70% (Fig. 4). To achieve further inhibition, much higher concentrations of HQNO are required.

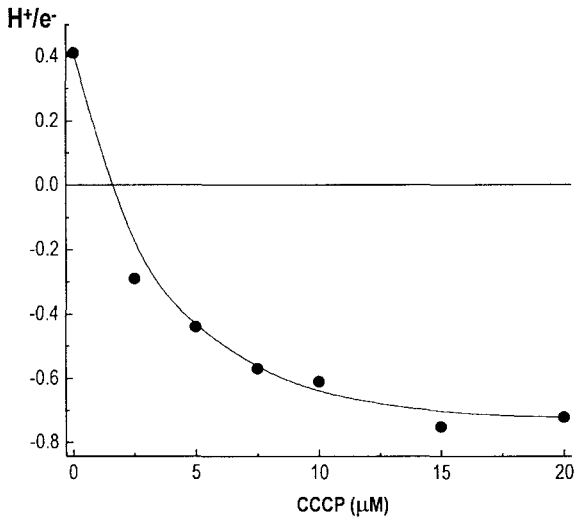
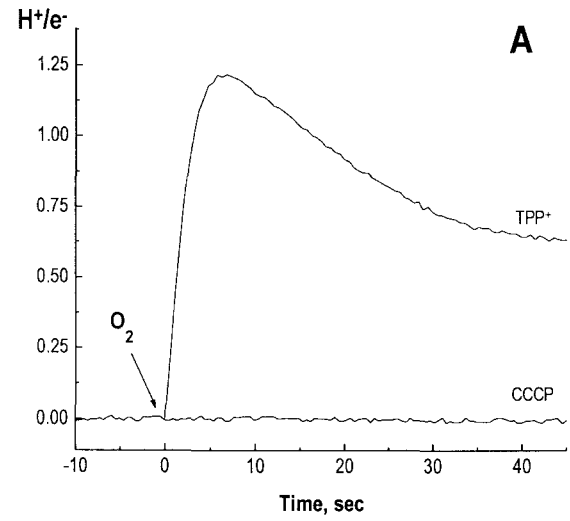


Fig. 2. The CCCP titration of measured H^+/e^- ratio in *V. alginolyticus* 138-2 cells oxidizing glycerol.



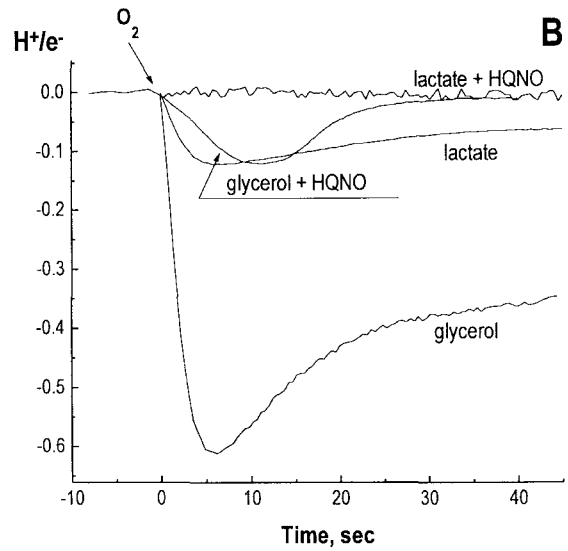


Fig. 3. (A) The H^+/e^- stoichiometries in *V. alginolyticus* mutant strain Nap1 lacking the Na^+-NADH : quinone oxidoreductase. Substrate, glycerol. Additions: 5 mM TPP^+, 15 μM CCCP. (B) The H^+/e^- stoichiometries in the *V. alginolyticus* 138-2 cells oxidizing D,L-lactate or glycerol in the presence of 15 μM CCCP. Effect of 5 μM HQNO.

The above data indicate that it is the Na^+-NADH : quinone oxidoreductase which is responsible for the CCCP-mediated H^+ influx when O_2 is added to anaerobic suspension of *V. alginolyticus* cells. This process: (i) is absent in the mutant strain lacking Na^+-NADH : quinone oxidoreductase; (ii) is sensitive to low concentrations of HQNO specifically inhibiting this enzyme [1,14]; and (iii) cannot be effectively supported by oxidation of D-lactate (a substrate which is oxidized in this bacterium in the NAD^+-independent fashion).

At least two facts suggest that, under the conditions employed, the bacterial membrane has a low permeability to ions other than H^+. (1) Without TPP^+ and CCCP, the O_2 pulse produces only a small pH change. (2) The rate of *decay* of the

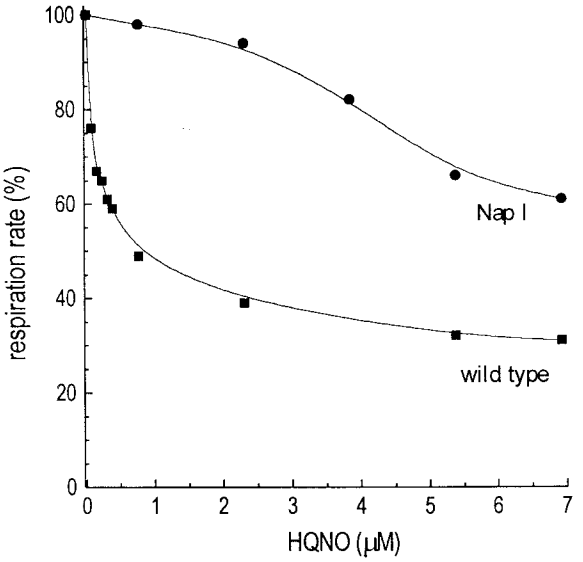


Fig. 4. The HQNO respiration inhibition in wild-type *V. alginolyticus* 138-2 cells and in the Napl mutant strain. Substrate, glycerol.

alkaline pH shift after the O_2 pulse in the presence of CCCP is much slower than the rate of *formation* of this pH change. Since in these experiments we used saturating CCCP concentrations, the observation in (2) indicates that penetration of ions other than H^+ are rate-limiting for the alkaline pH shift decay.

Thus, in samples with high CCCP concentrations, the membrane permeability for H^+ is much higher than for other ions so amount of the Na^+ ions extruded by the Na^+-NADH : quinone oxidoreductase should be equal to that of the H^+ ions taken up to discharge the Na^+ efflux-generated $\Delta\Psi$. In other words, experimentally measured Na^+/e^- ratio is equal to the Na^+/e^- ratio, i.e. 0.71. In fact, this value should be lower than the true Na^+/e^- stoichiometry of the Na^+-NADH : quinone oxidoreductase since, besides the Na^+-NADH : quinone oxidoreductase sensitive to low [HQNO], some O_2 consumption is due to operation of the non-coupled NADH : quinone oxidoreductase which is much more resistant to HQNO [14]. Contribution of this enzyme and NAD^+-independent primary dehydrogenases, according to the HQNO titration of respiration of *V. alginolyticus* cells, is $\approx 30\%$ of the total O_2 consumption (Fig. 4). This means that the true Na^+/e^- stoichiometry of the Na^+-NADH : quinone oxidoreductase is ≈ 1. This value is lower than the H^+/e^- stoichiometry for the *Escherichia coli* H^+-motive NADH : quinone oxidoreductase which we estimated in our previous work as equal to at least 1.5 [15]. Nevertheless, the measured Na^+/e^- stoichiometry is in line with Rich's schemes of the Na^+-motive NADH : quinone oxidoreductase mechanism [9,16].

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