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$\Delta\Psi$ and ΔpH generation by the H^+ pumps of the respiratory chain and ATPase in subcellular vesicles from marine bacterium *Vibrio alginolyticus*

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Succinate and glycerol 3-phosphate oxidation and coupled $\Delta\Psi$ generation by the respiratory chain of inside-out subcellular vesicles of *Vibrio alginolyticus* do not depend on Na^+ concentration and are not suppressed by submicromolar concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide, arresting Na^+-motive NADH:quinone reductase. When the H^+-permeability of membranes is diminished by adding Mg^{2+} and by *N,N'*-dicyclohexylcarbodiimide treatment, Na^+-independent NADH oxidation appears to be competent in the $\Delta\Psi$ generation. NADH and succinate oxidations and ATP-hydrolysis result in ΔpH formation (inside acidic) in media with K^+ and valinomycin. Na^+-dependent NADH oxidation also participates in $\Delta\bar{\mu}H^+$ generation because the inhibition of Na^+-motive NADH:quinone reductase (a medium with 2-heptyl-4-hydroxyquinoline *N*-oxide and without added Na^+) slows down ΔpH formation. It is assumed that the NADH-quinone and quinole-O_2 steps of the respiratory chain are Na^+- and H^+-motive, respectively. Since ATP hydrolysis by the *V. alginolyticus* vesicles supports uphill transport of Na^+ (Dibrov, P.A., Skulachev, V.P., Sokolov, M.V. and Verkhovskaya, M.L. (1988) FEBS Lett. 233, 355–358) as well as of H^+ (this paper), we may conclude that Na^+-cycle and H^+-cycle co-exist in the membrane of this bacterium.

Introduction

Membrane energetics of the marine alkalotolerant bacterium *Vibrio alginolyticus* is given considerable attention because it possesses the Na^+-motive NADH:quinone reductase [1]. The $\Delta\bar{\mu}_{Na^+}$ produced was found to be utilized to support motility [2,3], ATP synthesis [4] and solute transport [5,6]. On the basis of these data, the Na^+-cycle was postulated [2,7,8].

On the other hand, indications were obtained that primary H^+-pumps are also inherent in this bacterium. It was shown that an O_2 pulse leads to a CCCP-sensitive H^+efflux, the process being accelerated by the permeant cation, tetraphenylphosphonium [1]. The primary Na^+ pump was demonstrated in membrane preparations [3,9,10] and intact cells [1]. $\Delta\bar{\mu}H^+$ generation was, however, observed only in intact cells, except Na^+-independent $\Delta\Psi$ formation supported by the ATPase of subcellular vesicles [11]. Moreover, it was suggested that the NADH oxidase of *V. alginolyticus* can pump only Na^+ [10].

Here, we demonstrate $\Delta\bar{\mu}_{H^+}$ generation by the ATPase and the respiratory chain of subcellular vesicles. These data allow us to postulate, in addition to the Na^+-cycle, an H^+-cycle in the *V. alginolyticus* membrane.

Materials and Methods

The bacterial strain used was *V. alginolyticus* 138-2, which was kindly supplied by Dr. H. Tokuda (Chiba University, Chiba, Japan) Bacteria were grown in a synthetic medium [12] in the presence of 0.1% yeast extract and 100 mM succinate or 1% glycerol at pH 8.5 and $t = 37\,^\circ$C. Subcellular vesicles were isolated as described earlier [13]. For $\Delta\Psi$ measurements, the subcellular vesicles were washed and suspended in a medium containing 10 mM Hepes-Tris (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/4 mM dithiothreitol/ and bovine serum albumin (1 mg per ml). For ΔpH mea-

Abbreviations: $\Delta\bar{\mu}_{H^+}$, $\Delta\bar{\mu}_{Na^+}$, electrochemical gradients of H^+ and Na^+, respectively; $\Delta\Psi$, transmembrane electric-potential difference; ΔpH, transmembrane difference in concentrations of H^+; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PCB^-, phenyldicarbaundecaborane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PEP, phospho*enol*pyruvate.

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surements the solutions were supplemented with 50 mM K_2SO_4; 5 mM Mops-NaOH or Mops-Tris (pH 7.0) was used.

Oxygen consumption was measured polarographically. The energy-dependent accumulation of PCB^- by subcellular vesicles was used for $\Delta\Psi$ measurements. The PCB^- concentration was monitored with an asolectin-impregnated Teflon filter (Millipore, $\varnothing 5$ μm) [13]. Electric potential difference on the filter was measured by Ag|AgCl electrodes connected with a VA-J-51 electrometric voltmeter (G.D.R.) and a recorder. The protein concentration was 0.2–0.3 mg per ml.

The fluorescence of acridine orange, measured in a MPF-4 Fluorimeter ('Hitachi') with excitation at 493 nm and emission at 530 nm, is expressed in percentage quenching upon energization of subcellular vesicles.

The ammonium salt of NADH was obtained by chromatography on cellulose DE-52. The Tris salts of NAD^+, ADP, PEP and the ammonium salt of DL-glycerol 3-phosphate were obtained by passage through a Dowex 50-X8 column. The Na^+ content was analyzed by flame photometry. Protein was measured by the biuret method with bovine serum albumin as standard.

Results

Oxidase activities of the V. alginolyticus subcellular vesicles: dependence on Na^+ and inhibition by HQNO

It is known that NADH oxidation depends on Na^+, submicromolar concentrations of HQNO being inhibitory for the Na^+-dependent part of the activity [3]. We have confirmed this observation (Table I). It was also found that succinate oxidation is independent of Na^+ concentration (Table I) and is less sensitive to HQNO (Fig. 1). In other experiments, it was revealed that subcellular vesicles from *V. alginolyticus* grown on glycerol could oxidize glycerol 3-phosphate. This oxidation, independent of Na^+, proved to be resistant to micromolar HQNO (data not shown).

TABLE I

Effect of Na^+ upon NADH- and succinate oxidase activities

The incubation medium comprised 50 mM Tris-H_2SO_4 (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/10 mM $(NH)_4SO_4$/1 mM NADH (ammonium salt), or 5 mM succinate-Tris/0.14 mg protein of subcellular vesicles per ml. The Na^+ concentration in the medium without Na^+ additions is about 30 μM. When indicated, 10 mM Na_2SO_4 was added. The rate of respiration is expressed as nmol O_2/min per mg protein; $n = 3$.

Substrate	The rate of oxidation	
	$-Na_2SO_4$	$+Na_2SO_4$
NADH	130± 2	332±11
Succinate	364±15	347±16

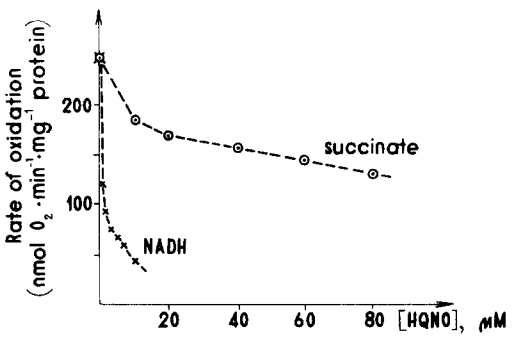

Fig. 1. Inhibition of succinate- and NADH-oxidase activities by HQNO. The incubation medium contained 50 mM Hepes-NaOH (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/5 mM succinate or 2 mM NADH/0.2 mg protein of subcellular vesicles per ml.

$\Delta\Psi$ generation coupled to oxidation of succinate and glycerol 3-phosphate

The oxidation of succinate was coupled to $\Delta\Psi$ generation (interior positive) (Fig. 2). Formation of $\Delta\Psi$ was inhibited by oxalacetate and cyanide, the protonophore CCCP, the ionophore gramicidin D and the permeant

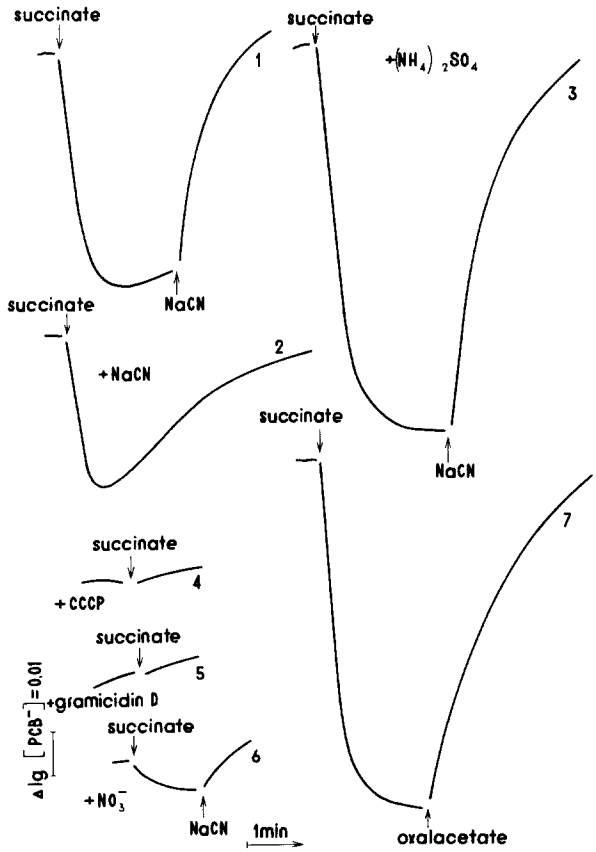

Fig. 2. Energy-dependent accumulation of PCB^- by subcellular vesicles coupled to succinate oxidation. The incubation medium contained 50 mM Hepes-NaOH (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/1 μM PCB^-/10 mM $(NH_4)_2SO_4$ (3–5, 7). Additions: 5 mM succinate/2 mM NaCN/5 μM CCCP/2 μM gramicidin D/50 mM $NaNO_3$/30 μM oxaloacetate.

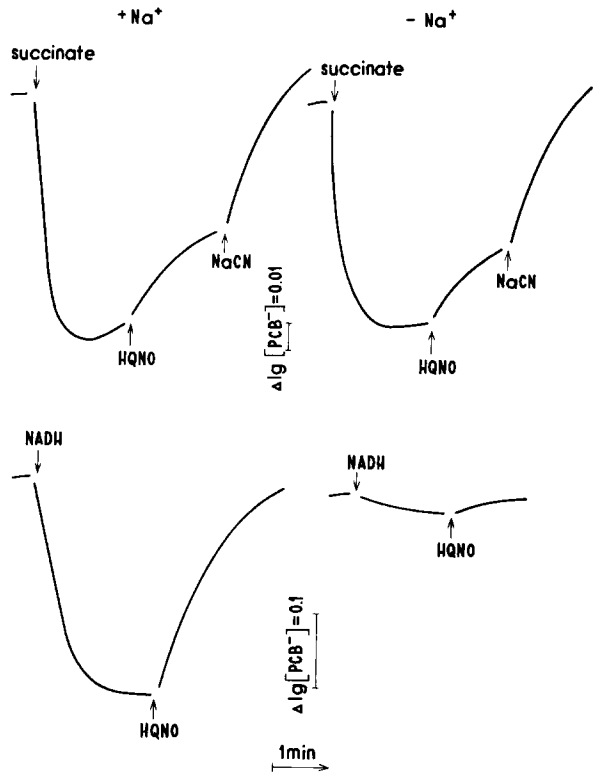

Fig. 3. Effect of Na^+ upon respiration-linked $\Delta\Psi$ generation. The incubation medium contained 50 mM Tris-H_2SO_4 (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/10 mM $(NH_4)_2SO_4$/1 μM PCB^-. Additions: 10 mM Na_2SO_4/5 mM succinate-Tris/1 mM NADH (ammonium salt)/1 μM HQNO/2 mM NaCN.

TABLE II

Effects of DCCD and Mg^{2+} on PCB^- accumulation supported by succinate oxidation

The incubation medium contained 50 mM Hepes-NaOH (pH 7.5)/0.1 M sucrose/10 mM $(NH_4)_2SO_4$/1 μM PCB^-. 5 mM succinate was added. Where indicated the incubation medium either was supplemented with 30 mM $MgSO_4$ or subcellular vesicles were preincubated for 10 min with 40 μM DCCD, or both $MgSO_4$ and DCCD were added. The energy-dependent changes of lg[PCB^-] were normalized and the maximum value corresponds to 100.

	Δlg[PCB^-] (arbitrary units)	
	$-MgSO_4$	$+MgSO_4$
−DCCD	4	14
+DCCD	100	88

anion nitrate. At the same time, ammonium sulfate markedly increased $\Delta\Psi$.

A decrease in the Na^+ concentration from 20 mM to 20 μM (the concentration in the medium without added Na^+) did not affect the succinate-dependent $\Delta\Psi$ (Fig. 3). Under these conditions, NADH-dependent $\Delta\Psi$ generation was drastically inhibited. 10 μM HQNO only partly reduced succinate-dependent $\Delta\Psi$. This $\Delta\Psi$ was not dependent on Na^+ at pH 8.5 (data not shown).

Previously, we have demonstrated that $\Delta\Psi$ formation supported by ATP hydrolysis or NADH oxidation was increased by Mg^{2+} at high concentrations. The optimal

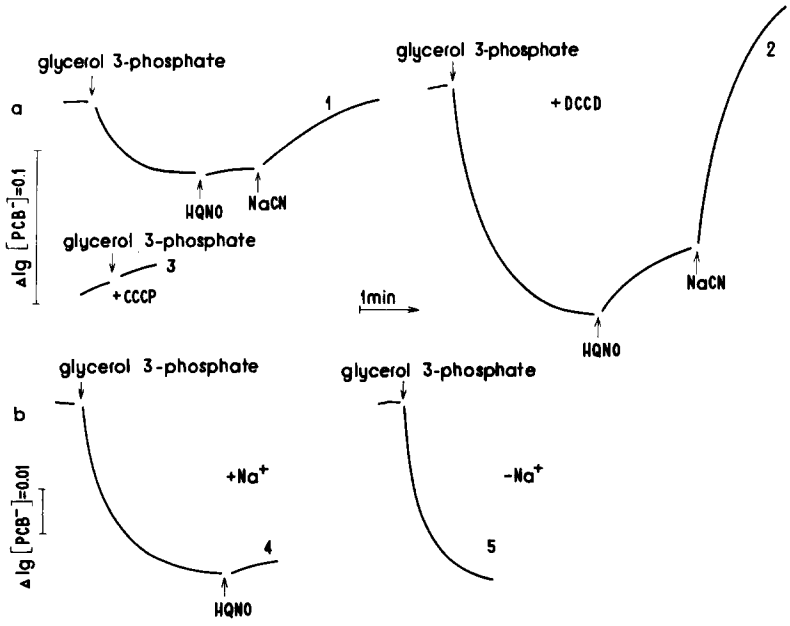

Fig. 4. $\Delta\Psi$ generation supported by glycerol 3-phosphate oxidation. (a) The incubation medium contained 50 mM Hepes-NaOH (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/1 μM PCB^-. Curve 2: subcellular vesicles were preincubated for 10 min with 40 μM DCCD. (b) The incubation medium contained 50 mM Tris-H_2SO_4 (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/10 mM $(NH_4)_2SO_4$/1 μM PCB^-. Curve 4: the incubation medium was supplemented with 10 mM Na_2SO_4. Additions: 5 mM glycerol 3-phosphate/5 μM HQNO/2 mM NaCN/2.5 μM CCCP.

TABLE III

Na^+-independent $\Delta\Psi$ generation supported by NADH oxidation

The incubation medium contained 50 mM Tris-H_2SO_4 (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/10 mM $(NH_4)_2SO_4$/1 μM PCB^-/1 mM NADH (ammonium salt). Subcellular vesicles (0.3 mg protein per ml) preincubated for 10 min with 40 μM DCCD. Addition: 10 mM Na_2SO_4/0.5 μM HQNO. The energy-dependent changes of $lg[PCB^-]$ were normalized and the maximum value of $\Delta lg[PCB^-]$ corresponds to 100.

Additions	$\Delta lg[PCB^-]$ (arbitrary units)
Na_2SO_4	100
–	30
HQNO	38
HQNO + Na_2SO_4	33

concentration of $MgSO_4$ was found to be 30 mM [11,13]. Mg^{2+} exerted the same effect on succinate-dependent $\Delta\Psi$ formation (Table II). It was also shown that the NADH-dependent $\Delta\Psi$ increased more if subcellular vesicles were preincubated with DCCD [11,13]. As shown in Table II, the same effect could be demonstrated for the succinate-dependent $\Delta\Psi$. These relationships were also inherent in the process of glycerol 3-phosphate oxidation-supported $\Delta\Psi$ generation (Fig. 4).

$\Delta\Psi$ generation coupled to NADH oxidation

As was suggested by Tokuda et al. [10], the NADH oxidation by oxygen forms $\Delta\bar{\mu}_{Na^+}$, not $\Delta\bar{\mu}_{H^+}$. However, under conditions favourable to $\Delta\Psi$ formation (high Mg^{2+}, DCCD treatment), a NADH-supported $\Delta\Psi$ was found to be formed in a medium without added Na^+. The response magnitude was about 30% of that in the Na^+-containing medium (Table III). HQNO did not reduce this Na^+-independent $\Delta\Psi$.

The above data seem to indicate that the *V. alginolyticus* subcellular vesicles are capable of generating $\Delta\Psi$ either by Na^+- or by H^+-pumps. The question arises: are the two mechanisms of $\Delta\Psi$ generation located in the same population of the subcellular vesicles? To answer this question, we studied the effect of Na^+-dependent $\Delta\Psi$ generation on succinate oxidation-driven $\Delta\Psi$ (Table IV). Preformed NADH-dependent $\Delta\Psi$ was shown to decrease a response to succinate addition by a factor of 5. At the same time, in the sample where Na^+-dependent $\Delta\Psi$ generation was inhibited by 1 μM HQNO, the succinate-induced PCB^- accumulation was almost as high as in the absence of NADH. HQNO at this concentration inhibits only Na^+-dependent, but not Na^+-independent NADH oxidation [3]. Thus, the reduction of the succinate-induced PCB^- accumulation seemed to be due to the action of the Na^+ pump. As NADH- and succinate-dependent $\Delta\Psi$ are not additive, one may assume that Na^+- and H^+-pumps are located in the membrane of the same subcellular vesicle.

TABLE IV

Effect of preformed NADH-dependent $\Delta\Psi$ on succinate-dependent $\Delta\Psi$

Incubation medium: 50 mM Hepes-NaOH (pH 7.5)/0.1 M sucrose/30 mM $MgSO_4$/10 mM $(NH_4)_2SO_4$/1 μM PCB^-. Additions: 5 mM succinate/1 mM NADH/1 μM HQNO. the energy-dependent changes of $lg[PCB^-]$ were normalized and the $\Delta lg[PCB^-]$ corresponds to 100 in case of the energization of subcellular vesicles only by succinate.

Additions	$\Delta lg[PCB^-]$ (arbitrary units)	$\Delta lg[PCB^-]$ increase after succinate addition
Succinate	100	100
NADH	247	
NADH + succinate	266	19
NADH	253	
NADH + HQNO	16	
NADH + HQNO + succinate	81	65

ΔpH generation coupled to succinate oxidation

ΔpH measurements with acridine orange showed that the terminal step(s) of respiratory chain and the ATPase

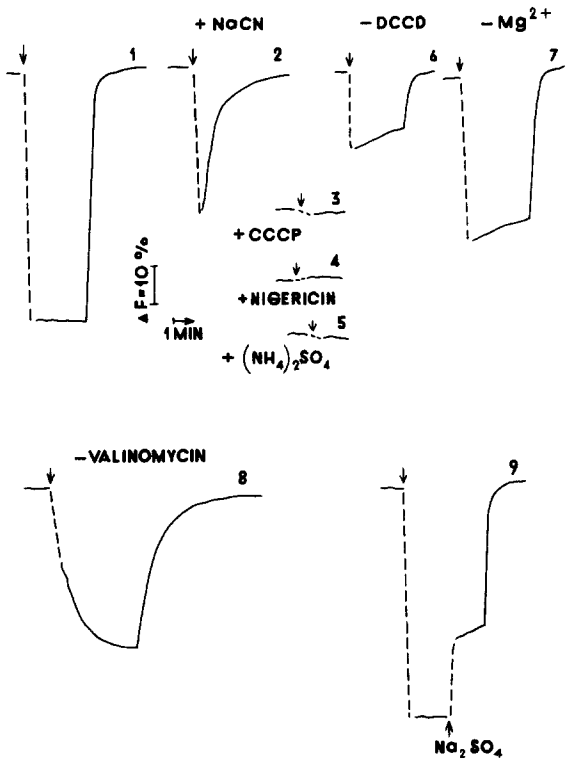

Fig. 5. Quenching of acridine orange fluorescence coupled to succinate oxidation. The incubation medium contained 30 mM Mops-KOH (pH 7.0)/0.1 M sucrose/30 mM $MgSO_4$/50 mM K_2SO_4/0.5 μM valinomycin/6 μM acridine orange. $MgSO_4$ or valinomycin was omitted from the medium in (7) and (8), respectively. The protein concentrations of subcellular vesicles were 0.75 mg per ml (1–8) and 0.51 mg per ml (9). Subcellular vesicles were preincubated for 20 min without DCCD (6) or with 13–27 μM DCCD. Additions: 1 mM NaCN/1 μM CCCP/0.7 μM nigericin/20 mM $(NH_4)_2SO_4$/10 mM Na_2SO_4. The increase in fluorescence to the initial level (1,6–9) was due to anaerobiosis. The arrows show succinate addition.

complex are competent in proton pumping. ΔpH formation coupled to succinate oxidation by subcellular vesicles is shown in Fig. 5 Fluorescence quenching following the succinate addition continued several minutes, and then the fluorescence increased to the initial level. The latter effect was due to oxygen exhaustion because the stirring of the solution restored the short-lived bleaching (not shown). The respiratory inhibitory cyanide, the protonophore CCCP, the K^+/H^+-antiporter nigericin and $(NH_4)_2SO_4$ were shown to prevent the energy-dependent bleaching of acridine orange fluorescence.

As in the case of $\Delta\Psi$ generation, the amplitude of ΔpH increased by DCCD and Mg^{2+} (Fig. 5, curves 1, 6, 7). Both the rate and magnitude of ΔpH formation were stimulated by valinomycin + K^+ (Fig. 5 curves 1 and 8). At the same time, Na^+ was not required for ΔpH formation coupled to succinate oxidation; in fact, the addition of Na^+ resulted in some decrease in the response amplitude (Fig. 5, curve 9).

It can be seen in Fig. 6 that monensin inhibited ΔpH formation in the Na^+-medium and had no effect in the medium without added Na^+. So, a Na^+/H^+-antiport mediated by monensin decreased the ΔpH. This fact contradicts a suggestion that a natural Na^+_n/H^+_m-antiporter ($n \leqslant m$) is involved in ΔpH formation by *V. alginolyticus* subcellular vesicles.

The NADH-dependent formation of ΔpH

The NADH oxidation was found to cause quenching of acridine orange fluorescence (Fig. 7). The effects of anaerobiosis, cyanide, CCCP, nigericin, ammonium

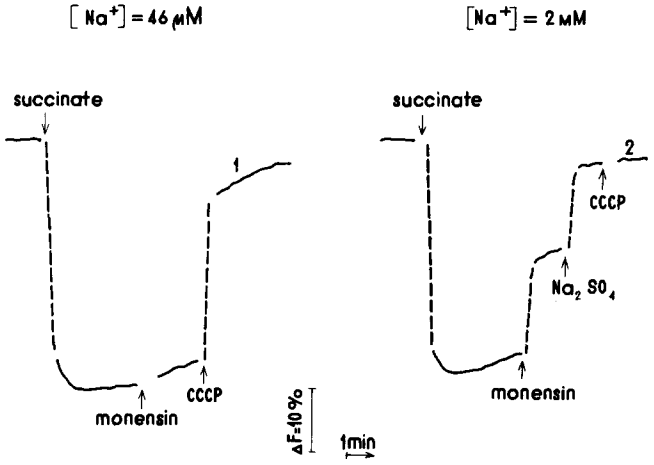

Fig. 6. Succinate-dependent ΔpH generation in the nitrate-containing medium. The effect of monensin. The incubation medium contained 30 mM Mops-Tris (pH 7.0)/0.1 M sucrose/30 mM $MgSO_4$/25 mM HNO_3-Tris/6 μM acridine orange/0.67 mg protein of subcellular vesicles per ml. Subcellular vesicles were preincubated for 20 min with 13 μM DCCD. Additions: 5 mM succinate-Tris/0.67 μM monensin/0.5 μM CCCP/6 mM Na_2SO_4. (1) Without added Na^+; Na^+ concentration is 46 μM; (2) with 1 mM Na_2SO_4.

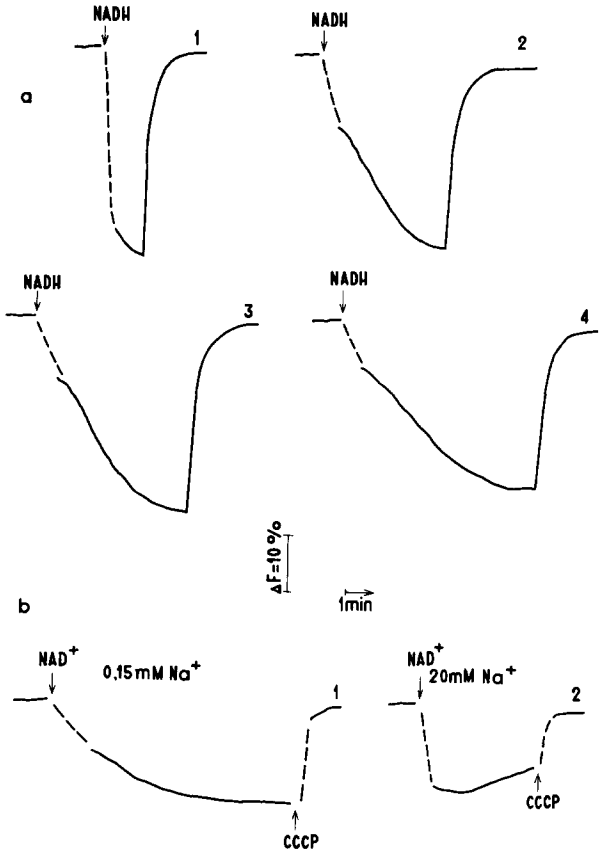

Fig. 7. NADH-dependent ΔpH formation: effect of HQNO (a) and Na^+ (b). (a) The incubation medium contained 30 mM Mops-KOH (pH 7.0)/0.1 M sucrose/30 mM $MgSO_4$/50 mM K_2SO_4/10 mM Na_2SO_4/0.5 μM valinomycin/6 μM acridine orange/1.3 mg protein of subcellular vesicles per ml. In samples 2, 3 and 4 0.5 μM, 1 μM and 2 μM HQNO were added. Subcellular vesicles were preincubated for 20 min with 27 μM DCCD. The fluorescence increase to the initial level was due to anaerobiosis. (b) The incubation medium contained 30 mM Mops-Tris (pH 7.0)/0.1 M sucrose/30 mM $MgSO_4$/50 mM K_2SO_4/0.5 μM valinomycin/6 μM acridine orange/6 μg alcohol dehydrogenase per ml/2 μl alcohol per ml/0.84 mg protein of subcellular vesicles per ml. Subcellular vesicles were preincubated for 20 min with 40 μM DCCD. Additions: 3 mM NAD^+/0.5 μM CCCP. (1) without added Na^+; Na^+ concentration was 150 μM. (2) with 10 mM Na_2SO_4.

sulfate, valinomycin, monensin, DCCD and Mg^{2+} on this process were similar to those in the case of succinate oxidation (data not shown). ΔpH could be formed in the medium without added Na^+ and in the presence of the inhibitor of Na^+-dependent NADH oxidase, HQNO but the rate of the process was significantly decreased (Fig. 7). Thus, both Na-independent and Na^+-dependent NADH oxidations were involved in the ΔpH formation.

Na^+-dependent NADH:quinone reductase is known to be more active at pH 8.5 than at neutral pH [1]. Since NADH and succinate oxidations cause the quenching of acridine orange fluorescence at pH 8.5 (data not shown), the H^+ pumps of the respiratory chain can also function under these conditions.

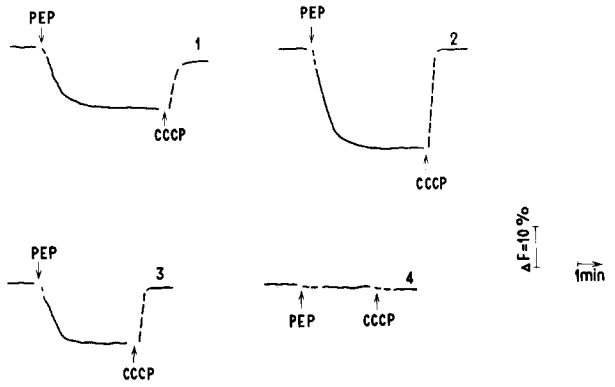

Fig. 8. ΔpH generation supported by ATP hydrolysis. 'Coupling' and inhibitory effects of DCCD. The incubation medium contained 30 mM Mops-Tris (pH 7.0)/0.1 M sucrose/30 mM $MgSO_4$/50 mM K_2SO_4/0.5 μM valinomycin/6 μM acridine orange/2 mM ADP/1 unit pyruvate kynase per ml/1.1 mg protein of subcellular vesicles per ml. Subcellular vesicles were preincubated for 20 min without DCCD (1) or with 13 μM (2), 27 μM (3), 80 μM (4) DCCD. Additions: 2 mM PEP-Tris/0.5 μM CCCP. Na^+ concentration was 150–200 μM.

ΔpH formation coupled to ATP hydrolysis

ATP hydrolysis was coupled to acidification of the interior of subcellular vesicles (Fig. 8). Since adenine nucleotides affect Acridine orange fluorescence, ΔpH formation was monitored with a coupled enzyme system and the reaction was started by PEP addition.

CCCP, nigericin and ammonium sulfate inhibited ΔpH formation (data not shown). 15 μM DCCD increased whereas 80 μM DCCD completely inhibited ΔpH formation (Fig. 8). At the same time, 80 μM DCCD increased ΔpH under the energization of vesicles' membranes by succinate oxidation (not shown). Thus, the inhibitory action of high DCCD concentration on ΔpH formation is due to the inhibition of an ATPase.

The data shown in Fig. 8 have been obtained in experiments carried out in a medium with the Na^+ content lower than 0.2 mM. The addition of 10 mM Na_2SO_4 decreases the ΔpH magnitude by 25–30% (data not shown).

Discussion

The following data indicate operation of an H^+ pump, rather than Na^+ pump, in the succinate oxidase. (i) The rate of succinate oxidation and a $\Delta\Psi$ generation are independent of Na^+ at concentrations from 20–30 μM to 20 mM. (ii) Succinate oxidation is coupled to ΔpH formation (acidic inside) in the medium without added Na^+. Na^+ addition decreases ΔpH. (iii) Ammonium salt increases $\Delta\Psi$, and decreases ΔpH. (iv) Valinomycin stimulates the ΔpH formation. (v) The Na^+/H^+-antiporter, monensin, inhibits a ΔpH formation in a medium containing Na^+ and no K^+.

Glycerol 3-phosphate oxidation accompanied by $\Delta\Psi$ generation shares common features with succinate-dependent one. In both cases, oxidation and electrogenesis are independent of Na^+ and resistant to micromolar HQNO. These data suggest that H^+ pump(s) are localized in a segment of the respiratory chain which is common for succinate and glycerol 3-phosphate oxidases, i.e., in the cytochrome system.

In the medium without added Na^+, a NADH-dependent $\Delta\Psi$ formation was observed. The $\Delta\Psi$ generation was not arrested by submicromolar HQNO. When K^+ and valinomycin or nitrate were present, a ΔpH was formed. Thus, Na^+-dependent NADH oxidase can generate $\Delta\bar{\mu}_{H^+}$. Simultaneously, Na^+-motive NADH oxidation was involved in $\Delta\bar{\mu}_{H^+}$ generation, for the inhibition of Na^+-dependent NADH:quinone reductase (HQNO, medium without added Na^+) slowed down ΔpH formation. The observations are in accordance with the location of Na^+- and H^+-pumps in NADH:quinone reductase and in the cytochrome system respectively. These results are summarized in Fig. 9.

Since the authors of the earlier work [10] could not detect the accumulation of H^+ in subcellular vesicles, they concluded that the NADH oxidase of *V. alginolyticus* was coupled only to the Na^+ pumping. For the first time, $\Delta\bar{\mu}_{H^+}$ generation by subcellular vesicles from *V. alginolyticus* was the observation when ATP hydrolysis in the low Na^+ medium was studied [11]. ATP-driven ΔpH formation (Fig. 8) in the medium without added Na^+ corroborates the protonic nature of the pump. The ATP-driven $\Delta\Psi$ is greatly influenced by Mg^{2+}, the optimal concentration of Mg^{2+} being about 30 mM [11,13]. DCCD is another and more effective 'coupling'

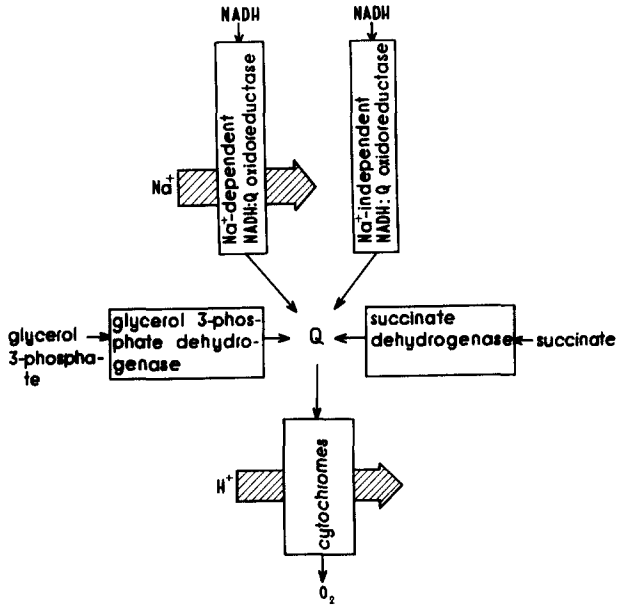

Fig. 9. A tentative scheme of respiratory chain of *V. alginolyticus*. The thin arrows show the transfer of electrons, the thick arrows indicate the transmembrane transport of Na^+ and H^+.

agent ([3,11], Table II, Figs. 4 and 5). $\Delta\Psi$ and ΔpH monitoring under coupling conditions enables us to show the existence of H^+ pumps linked to the respiratory chain and the ATPase.

DCCD exerts a coupling effect on $\Delta\Psi$ and ΔpH in the absence Na^+, K^+ and Mg^{2+} (this work and [3,11]). Therefore, the effect of DCCD may be accounted for the reduction of passive proton leakage. It seems that Mg^{2+} and DCCD exert the same action on the conductivity of the membranes because in the presence of DCCD practically the same response was observed in media with and without $MgSO_4$ ([11] and Table II). Probably, Mg^{2+} and DCCD diminish proton leakage through an ATPase complex. It should be noted that DCCD couples Na^+ transport by the Na^+-ATPase of subcellular vesicles from *V. alginolyticus* as well [14]. The common Na^+- and H^+-path through the ATPase complex agrees with the ideas recently published by Boyer [15].

The demonstration of H^+ pumps associated with the respiratory chain and the ATPase allows to postulate a H^+-cycle for *V. alginolyticus*. Since earlier the Na^+-cycle for the oxidative phosphorylation of *V. alginolyticus* was shown [4], the question arises about the regulation of enzymes transferring Na, H^+ or both. For example, at alkaline pH a Na^+ concentration in the cells of *V. alginolyticus* is lower, but H^+ concentration is higher than outside [16]. For generators with the same stoichiometry of H^+ (Na^+) per $2e^-$ or ATP, $\Delta\bar{\mu}_{Na^+} > \Delta\bar{\mu}_{H^+}$. In this case only H^+-generators can be functioning under conditions close to the respiratory control state. Another problem is the simultaneous operation of Na^+- and H^+-dependent ATP synthase, because the H^+-dependent reaction may proceed in the reverse direction under these conditions and lead to the futile hydrolysis of ATP and to the disturbing of pH-homeostasis.

The coexistence of H^+- and Na^+-energetics is a common feature for several bacteria possessing Na^+ pumps. So it was suggested that the Na^+ and H^+-pumps operate in NADH:quinone reductase and quinone oxidase steps of the respiratory chain of *V. costicola* [17]. The same suggestion was made for bacterium Ba_1 [18]. *Streptococcus faecalis* has Na^+-ATPase and H^+-ATPase [19]. The $\Delta\bar{\mu}_{Na^+}$- and $\Delta\bar{\mu}_{H^+}$-generating reactions were demonstrated for a methanogenic *Methanosarcina barkeri* [20]. The possibility of a H^+-cycle and Na^+-cycle operation was also shown for *V. parahaemolyticus* [21].

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References

1 Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014.
2 Chernyak, B.V., Dibrov, P.A., Glagolev, A.N., Sherman, M.Yu. and Skulachev, V.P. (1983) FEBS Lett. 164, 38–42.
3 Dibrov, P.A., Kostyrko, V.A., Lazarova, R.L., Skulachev, V.P. and Smirnova, I.A. (1986) Biochim. Biophys. Acta 850, 449–457.
4 Dibrov, P.A., Lazarova, R.L., Skulachev, V.P. and Verkhovskaya, M.L. (1986) Biochim. Biophys. Acta 850, 458–465.
5 Tokuda, H., Sugasawa, M. and Unemoto, T. (1982) J. Biol. Chem. 257, 788–794.
6 Kakinuma, Y. and Unemoto, T. (1985) J. Bacteriol. 163, 1293–1295.
7 Glagolev, A.N., Dibrov, P.A., Skulachev, V.P. and Sherman, M.Yu. (1984) Biologicheskie Membrany 1, 27–32.
8 Skulachev, V.P. (1985) Biokhimiya 50, 179–183.
9 Tokuda, H. (1984) FEBS Lett. 76, 125–128.
10 Tokuda, H., Udagawa, T. and Unemoto, T. (1985) FEBS Lett. 183, 95–98.
11 Smirnova, I.A., Vulfson, E.N. and Kostyrko, V.A. (1987) FEBS Lett. 214, 343–346.
12 Tokuda, H., Nakamura, T. and Unemoto, T. (1981) Biochemistry 20, 4198–4203.
13 Smirnova, I.A., Vulfson, E.N. and Kostyrko, V.A. (1987) Biokhimiya 52, 1939–1947.
14 Dibrov, P.A., Skulachev, V.P., Sokolov, M.V. and Verkhovskaya, M.L. (1988) FEBS Lett. 233, 355–358.
15 Boyer, P.D. (1988) Trends Biochem. Sci. 13, 5–7.
16 Nakamura, T., Tokuda, H. and Unemoto, T. (1984) Biochim. Biophys. Acta 776, 330–336.
17 Udagawa, T., Unemoto, T. and Tokuda, H. (1986) J. Biol. Chem. 261, 2616–2622.
18 Ken-Dror, S., Lanyi, J.K., Schobert, B., Silber, B. and Avi-Dor, Y. (1986) Arch. Biochem. Biophys. 244, 766–772.
19 Kakinuma, Y. (1987) J. Bacteriol. 169, 3886–3890.
20 Muller, V., Winner, C. and Gottschalk, G. (1988) Eur. J. Biochem. 178, 519–525.
21 Sakai, Y., Moritani, C., Tsuda, M. and Tsuchiya, T. (1989) Biochim. Biophys. Acta 973, 450–456.