

The ATP-driven primary Na^+ pump in subcellular vesicles of *Vibrio alginolyticus*

P.A. Dibrov, V.P. Skulachev, M.V. Sokolov and M.L. Verkhovskaya

Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

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Subcellular vesicles of *Vibrio alginolyticus* hydrolyze ATP and accumulate Na^+ in an ATP-dependent fashion. The Na^+ uptake is (i) strongly stimulated by $\Delta\psi$ -discharging agents, i.e., the protonophorous uncoupler CCCP or valinomycin + K^+ and (ii) arrested by DCCD at a concentration strongly inhibiting ATP hydrolysis. Lower concentrations of DCCD stimulate the Na^+ accumulation supported by ATP hydrolysis as well as by NADH oxidation. It is concluded that there is an electrogenic DCCD-sensitive Na^+ -ATPase in the cytoplasmic membrane of *V. alginolyticus*.

Na^+ -ATPase; Na^+ bioenergetics; Bacterial vesicle; (*Vibrio alginolyticus*)

1. INTRODUCTION

In the marine alkalotolerant *Vibrio alginolyticus*, the electrochemical Na^+ potential difference ($\Delta\mu\text{Na}$) can be generated by Na^+ -motive NADH-quinone reductase [1–3] and consumed by systems carrying out (i) accumulation of metabolites via Na^+ , the metabolite symporters [4], (ii) rotation of the flagellum by the Na^+ motor [5,6] and (iii) ATP synthesis [7,8]. The latter effect, demonstrated in intact cells, was postulated to involve a Na^+ -ATP-synthase which, if reversible, may be regarded as Na^+ -ATPase. Below we shall describe experiments indicating that in the *V. alginolyticus* membrane, there is an ATP-dependent mechanism competent in the electrogenic transport of Na^+ .

Correspondence address: P.A. Dibrov, Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Abbreviations: $\Delta\mu\text{H}$ and $\Delta\mu\text{Na}$, electrochemical H^+ and Na^+ potential difference, respectively; CCCP, *m*-chlorocarbonyl-cyanide phenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide

2. MATERIALS AND METHODS

The *V. alginolyticus* strain 138-2, kindly supplied by Professor H. Tokuda (Chiba University, Chiba, Japan), was grown aerobically at 37°C in a salt medium [3] in the presence of 0.5% glucose and 0.5% peptone, pH 8.6. At the late-logarithmic phase, the cell suspension was diluted two-fold with the same medium supplemented with penicillin (10^6 units $\times$ l^{-1}) and incubated for 70 min at 37°C. The obtained spheroplasts were sedimented at $7500 \times g$ for 10 min and washed with a solution of 25 mM Tris- H_2SO_4 , 30 mM MgSO_4 , 5 mM Na_2SO_4 and 150 mM K_2SO_4 , pH 7.5 (medium A), or 50 mM Tricine, 30 mM MgSO_4 , 5 mM Na_2SO_4 and 250 mM K_2SO_4 , pH 8.2 (medium B). The final spheroplast sediment was suspended in the same media supplemented with a serine proteinase inhibitor, 0.5 mM phenylmethylsulfonyl fluoride. The suspension was sonicated in an ultrasonic UZDN-2T desintegrator for 10 s, four times with 1 min intervals, 0°C, at a frequency of 22 kHz, 4×10^{-5} A current and maximal resonance. Intact and partially destroyed spheroplasts were removed by centrifugation at $1200 \times g$ for 10 min at 2°C. Subcellular vesicles were sedimented from the supernatant ($48500 \times g$, 1 h, 2°C). The precipitate was suspended in medium A or B, using a glass homogenizer with a Teflon pestle and stored at 0°C. The final protein concentration in the stock solution was $10 \text{ mg} \cdot \text{ml}^{-1}$.

Na^+ transport was measured with the use of gel-filtration and centrifugation procedures [10]. The process was initiated by adding 10 mM substrate (ATP or NADH) to the suspension of subcellular vesicles. To stop the reaction, the suspension (0.05 ml) was centrifuged in a gel-filtration column ($D = 10 \text{ mm}$) with Sephadex G-50 coarse. As a buffer, a solution containing 5 mM Tris- H_2SO_4 and 120 mM MgSO_4 , pH 8.5, was

used. The eluate was diluted 10-fold so as to measure Na^+ with a PFM flame photometer.

3. RESULTS

In agreement with the observation of Tokuda et al. [11] on everted subcellular *V. alginolyticus*

vesicles, we have found that NADH oxidation is coupled to Na^+ uptake by the vesicles, the process being stimulated by valinomycin and inhibited by monensin (fig.1A). Under the same conditions, however, no measurable ATP-dependent Na^+ uptake was observed (not shown).

Further experiments showed that 2×10^{-5} M

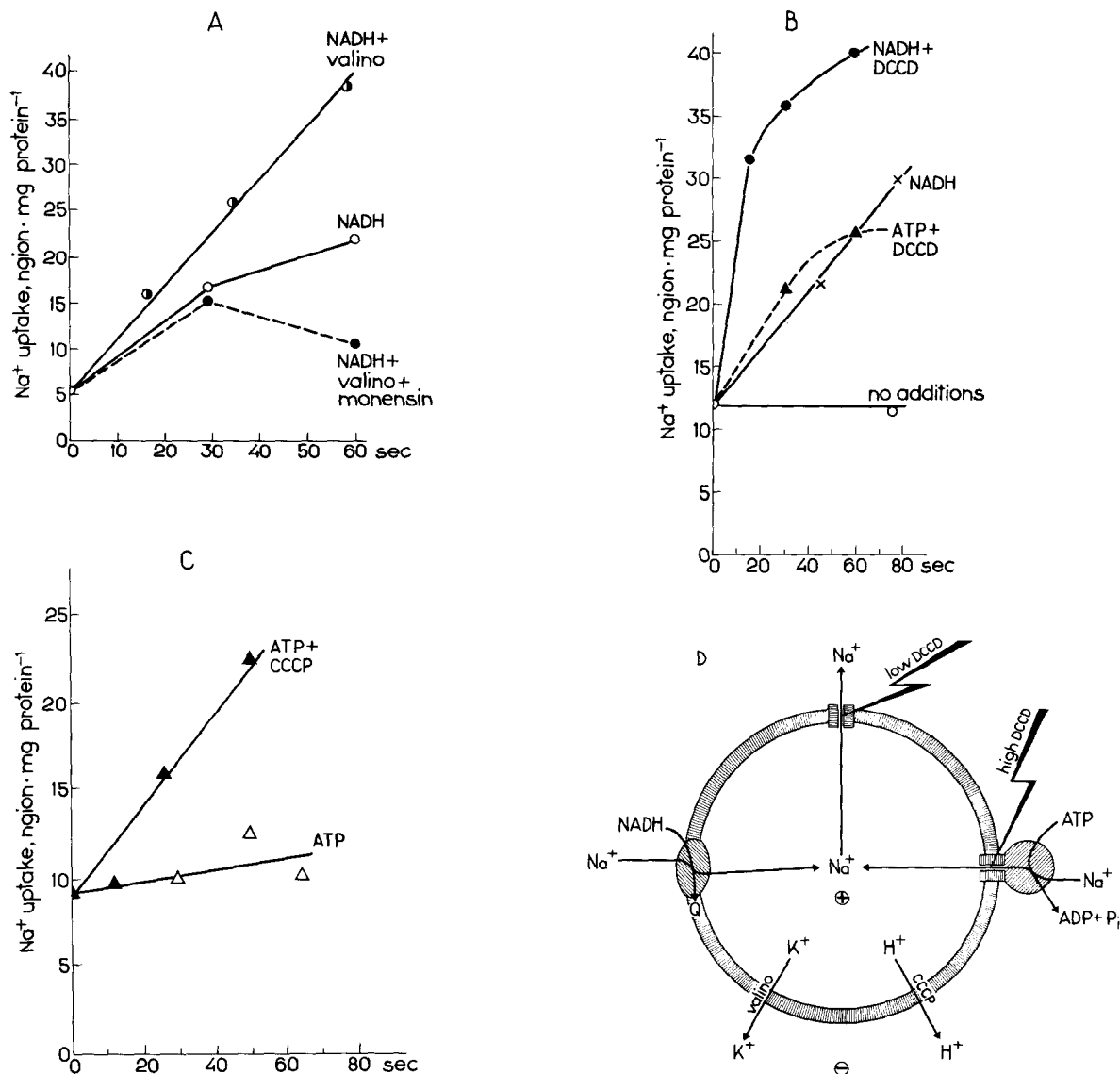


Fig.1. Na^+ uptake supported by NADH oxidation or ATP hydrolysis in *V. alginolyticus* subcellular vesicles. (A) Medium A; (B,C) medium B. (B) 5×10^{-6} M valinomycin in all the samples; (C) vesicles were preincubated with 2×10^{-5} M DCCD for 10 min (similar DCCD treatment was applied to samples with DCCD in B). Where indicated, the following additions were made at zero time: 10 mM NADH, 5 mM ATP, 2×10^{-6} M valinomycin, 4×10^{-6} M monensin and 2×10^{-5} M CCCP. (D) A scheme illustrating the interplay of the energy-linked and passive ion fluxes in the vesicles.

Table 1

DCCD effect on ATPase activity and ATP-dependent Na⁺ uptake by *V. alginolyticus* subcellular vesicles

[DCCD] (μ M)	Preincuba- tion (min)	ATPase (nmol ATP · mg protein ⁻¹ · min ⁻¹)	Na ⁺ uptake (ng ion · mg protein ⁻¹ · min ⁻¹)
—	—	365	5
20	10	275	35
200	20	90	0

DCCD (3 nmol · mg protein⁻¹) strongly stimulated the NADH-oxidation supported Na⁺ transport; addition of ATP also resulted in an Na⁺ uptake which proceeded at a somewhat lower rate than in the NADH-containing sample (fig. 1B). A higher (2×10^{-4} M) concentration of DCCD inhibited the ATP-dependent Na⁺ accumulation and the ATPase activity (table 1).

Just as in the case of respiration-linked Na⁺ uptake, the ATP effect was greatly stimulated by valinomycin and abolished by monensin. The protonophorous uncoupler CCCP was found to effectively substitute for valinomycin as an activator of the ATP-dependent Na⁺ transport. Fig. 1C shows that in the absence of valinomycin, no Na⁺ uptake was induced by the ATP addition to the vesicles treated with a small amount of DCCD. Such an uptake occurred when 2×10^{-5} M CCCP was present in the incubation mixture.

4. DISCUSSION

The above data are summarized in fig. 1D. It is shown that Na⁺ can be transported into everted vesicles by means of Na⁺-motive NADH-quinone reductase of Na⁺-motive ATPase. Such transport results in a positive charging of the vesicle interior. The formed $\Delta\psi$, which prevents large-scale Na⁺ accumulation, can be discharged by an efflux of K⁺ or H⁺ in the presence of valinomycin or CCCP, respectively. Apparently the procedure of preparation of vesicles caused a partial loss of the catalytic parts of the Na⁺-ATPase, whereas the Na⁺-transporting parts remained in the membrane and increased its Na⁺ conductance. This conductance seems to be sensitive to low concentrations of DCCD which prevents the leakage of accumulated Na⁺. A high amount of DCCD strongly inhibits

ATPase and therefore arrests the ATP-dependent Na⁺ uptake. In this context, it should be mentioned that Na⁺-ATPase of *Propionigenum modestum* proved to be a DCCD-sensitive enzyme of the subunit composition similar to that of the F₀F₁ type H⁺-ATPase [12].

An interesting problem is the relation of the above described ATP-dependent Na⁺ to the H⁺-ATPase activity previously described by our group in the *V. alginolyticus* vesicles [13,14]. The ATP-linked H⁺ transport is also stimulated by low DCCD, inhibited by high DCCD and stimulated by valinomycin. However, CCCP completely abolished the H⁺ uptake by vesicles. This fact is in contrast to the above data on the stimulation of Na⁺ transport by the same protonophore. The latter observation excludes any explanation of the ATP-supported Na⁺ transport as a consequence of utilization of the $\Delta\bar{\mu}H$ produced by H⁺-ATPase.

Three possibilities may be considered to account for the ATP-linked Na⁺ and H⁺ pumps in *V. alginolyticus*: (i) there are two DCCD-sensitive ATPases in the same membrane, one for Na⁺ and the other for H⁺; (ii) a single ATPase can transport alternatively Na⁺ or H⁺; (iii) a single ATPase is competent in the symport of Na⁺ and H⁺.

It may be noted that Na⁺-ATPase of *P. modestum* transports H⁺ when Na⁺ is absent [15] and animal Na⁺/K⁺-ATPase performs an H⁺/K⁺ antiport at acidic pH in a Na⁺-free medium [16]. According to Boyer, substitution of Na⁺ for H⁺ can be explained assuming that the cation-binding sites are organized as crown ethers coordinating Na⁺ or H₃O⁺ [17].

The functional significance of the existence of two primary ATP-driven pump activities, one dealing with H⁺ and the other with Na⁺, may mean that *V. alginolyticus* can survive in both neutral and alkaline conditions. At neutral pH, the proton cycle can be operative, which involves the H⁺-motive respiratory chain and H⁺-ATP-synthase. At alkaline pH, when ΔpH and $\Delta\psi$ are oppositely directed, the sodium cycle is actuated, with the Na⁺-motive respiratory chain and Na⁺-ATP-synthase being involved. In agreement with this reasoning, it was shown in our group that the *V. alginolyticus* respiratory chain, besides the $\Delta\bar{\mu}Na$ -generating NADH-quinone reductase, includes the $\Delta\bar{\mu}H$ generator(s) between quinol and O₂ [18].

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