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The effect of F_0 inhibitors on the *Vibrio alginolyticus* membrane ATPase

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The inhibition of membrane ATPase from the marine alkalotolerant bacterium *Vibrio alginolyticus* by DCCD, triphenyltin and venturicidin was studied. DCCD proved to be an irreversible inhibitor, while venturicidin and triphenyltin produced a reversible inhibitory effect. The DCCD-binding proteolipid was identified in the membrane preparations. The effect of the inhibitors on ATPase activity and ATP-dependent Na^+-transport in *V. alginolyticus* subcellular vesicles is discussed.

Bacterial F_0F_1-ATPase; $Na^+(H^+)$-ATPase; *Vibrio alginolyticus*

1. INTRODUCTION

We reported previously some characteristics of the membrane ATPase from the marine alkalotolerant bacterium *Vibrio alginolyticus* [1]. This enzyme proved to belong to the F_0F_1-type. We purified the F_1-component and determined the N-terminal amino acid sequences of its major subunits, which were found to be highly homologous to those of *Escherichia coli* F_1-ATPase [2]. An independent study, undertaken by Krumholz et al. [3] has shown that the sequences and organization of the *V. alginolyticus* and *E. coli* ATPase operons are very similar. The *V. alginolyticus* operon was cloned in *E. coli* and the ATPase complex was isolated. Proteoliposomes with such ATPase proved to be competent in H^+-pumping [4]. These observations, in fact, confirmed the data obtained in our group by Smirnova et al. [5,6] that the *V. alginolyticus* inside-out subcellular vesicles pump H^+ in an ATP-dependent fashion. The process is DCCD-sensitive.

A parallel study, carried out in our laboratory by Dibrov et al. [7], showed that the same subcellular vesicles can also pump Na^+, the process being coupled to ATP-hyrolysis and strongly stimulated by a protonophorous uncoupler. In the whole cells, indications were obtained, that the Na^+-driven ATP-synthase is operative under alkaline conditions [8,9]. In the inside-out vesicles, uncoupler-stimulated ATP-synthesis supported by an artificially imposed ΔpNa was shown (P.A. Dibrov, V.P. Skulachev and M.V. Sokolov, unpublished results).

Since no other ATPase operon was found in the *V. alginolyticus* genome [3], it was suggested [9,10] that one and the same ATPase can transport either H^+ or Na^+ as was found with the *Propionigenium modestum* ATPase by Dimroth's group [11].

In this paper we have studied the effect of three inhibitors (DCCD, triphenyltin and venturicidin) on the F_0-component of the F_0F_1-ATPase from *V. alginolyticus*.

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide

2. MATERIALS AND METHODS

Vibrio alginolyticus was grown aerobically in a medium, containing 0.5 M NaCl, 100 mM succinate, 10 mM KCl, 5 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 10 mM Tris-HCl, pH 8.5, 0.1% yeast extract. Subcellular vesicles were prepared as described elsewhere [1].

ATPase activity was measured by monitoring the NADH oxidation in the presence of an ATP-regenerating system with pyruvate kinase and lactate dehydrogenase [12].

Labelling with [^{14}C]DCCD was performed overnight at 0°C, the inhibitor concentration being equal to 5 nmol/mg protein. Membranes were pelleted, washed 4 times with 90% acetone and extracted with a chloroform/methanol mixture (2:1 v/v). The extract was dried under nitrogen and dissolved in the sample buffer.

SDS-electrophoresis was performed essentially according to Laemmli [13]. The separating gel contained 15–25% polyacrylamide (linear gradient). Cyanogen bromide cleavage fragments of myoglobin produced by LKB (Sweden) were used as molecular weight markers. After electrophoresis, the gel was sliced in 2 mm pieces which were incubated overnight in 1 ml of 30% hydrogen peroxide, and then radioactivity was counted in a liquid scintillation counter. Silver staining was performed as described by Morrissey [14]. Protein was assayed by the Lowry method [15] using bovine serum albumin as standard. Tris, Mes, Hepes, ATP, venturicidin were from Sigma (USA); DCCD and triphenyltin was from Serva (Germany); [^{14}C]DCCD (specific activity 57 Ci/mol) was from Amersham (UK); and all other chemicals were of analytical grade.

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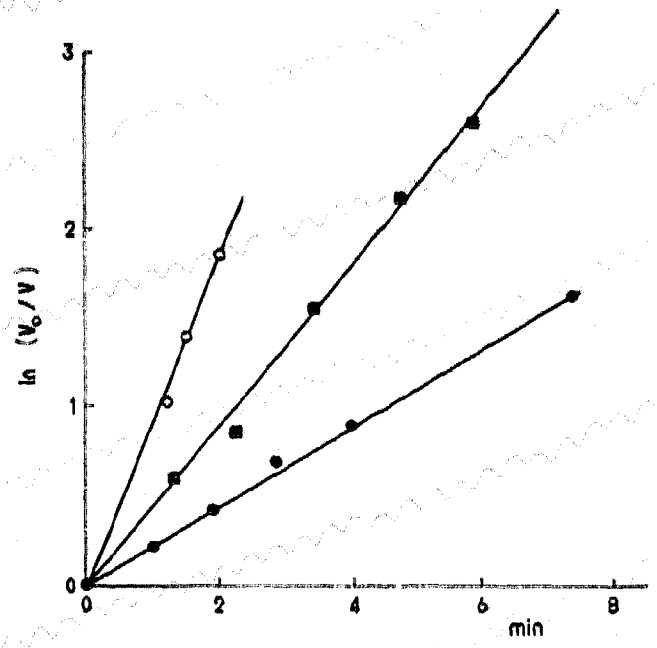

Fig. 1. Kinetics of the DCCD inhibition of ATPase activity in the *V. alginolyticus* membrane vesicles. The vesicles were preincubated with DCCD for the indicated period of time at pH 7.6 and then ATPase activity was measured. v_0, the rate of the ATPase reaction without DCCD; v, the reaction rate at a given time after the addition of DCCD. ○, 60 μM DCCD; ■, 30 μM DCCD; ●, 15 μM DCCD.

3. RESULTS AND DISCUSSION

DCCD was previously found to be a potent inhibitor of *V. alginolyticus* ATPase [1]. Fig. 1 shows that its inhibitory effect on the ATPase activity of *V. alginolyticus* subcellular vesicles obeys the first order kinetics, the rate constant being equal to 14.6 ± 0.5 $mM^{-1} \cdot min^{-1}$ at pH 7.6. The pH-dependence of the rate constant of the enzyme inactivation is shown in Fig. 2. Na^+ ions had no effect on the magnitude or pH-dependence of the rate constant (not shown).

DCCD is known to inhibit bacterial F_oF_1-ATPases reacting with the smallest F_o subunit (proteolipid) [16]. The results presented in Fig. 3 show that [^{14}C]DCCD is bound to a low molecular mass protein that could be extracted with chloroform/methanol. SDS-electrophoresis of the extract revealed a single peak of radioactivity below the 10 kDa region (Fig. 3). We were unable to observe any Coomassie binding in this region, but silver staining revealed a diffuse band, corresponding to the radioactivity peak. This band was also specifically revealed by Western blotting with antibodies against the *E. coli* subunit c, though reaction of the *V. alginolyticus* proteolipid was faint compared to that of the *E. coli* subunit c.

We have also studied the effect of 2 other F_o inhibitors, triphenyltin and venturicidin. Both inhibited the *V. alginolyticus* ATPase at rather low concentrations (Fig. 4). The effect of triphenyltin and venturicidin was shown to be reversible. Dithiothreitol abolished the triphenyltin inhibition. Triphenyltin did not prevent the DCCD-binding.

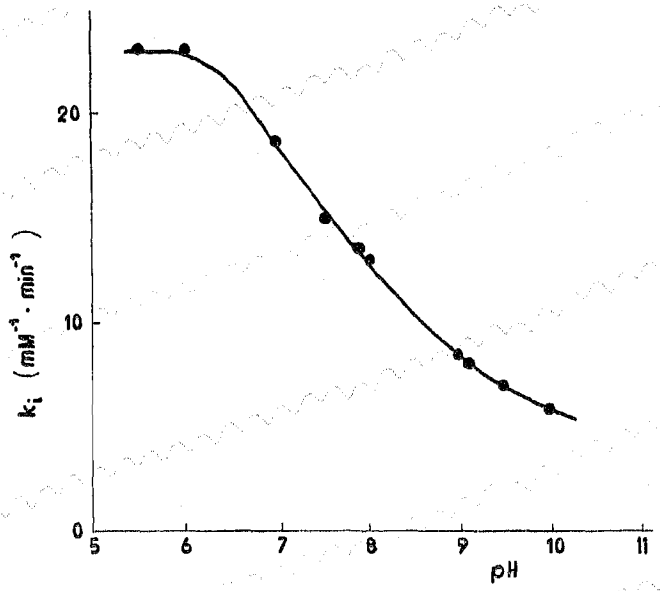

Fig. 2. pH-dependence of the rate constant of ATPase inhibition (k_i) by DCCD.

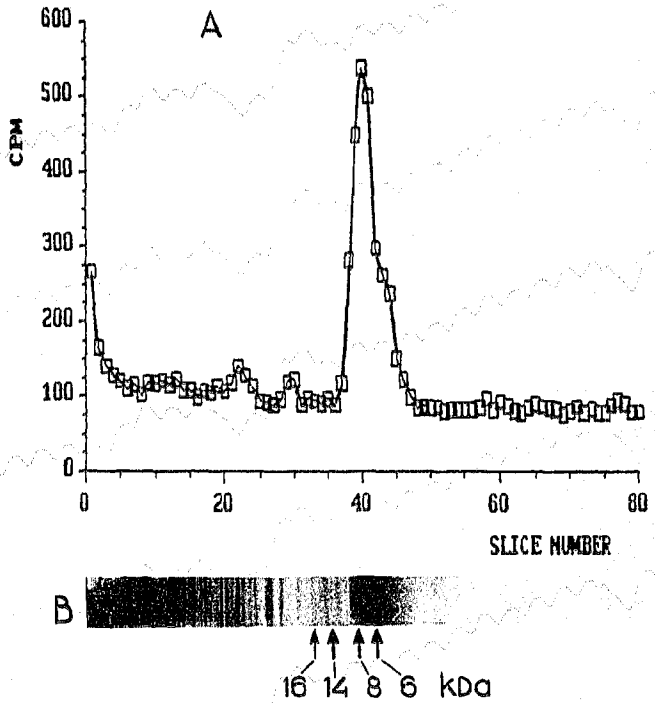

Fig. 3. Fractionation of the chloroform/methanol extract of [^{14}C]DCCD-treated vesicles by SDS-electrophoresis. (A) Distribution of the radioactivity in the gel. (B) Silver stained gel. Positions of the markers of known molecular weights are shown by arrows. Exact molecular weights of the markers as given by the manufacturer were 16.949 kDa, 14.404 kDa, 8.159 kDa, 6.214 kDa.

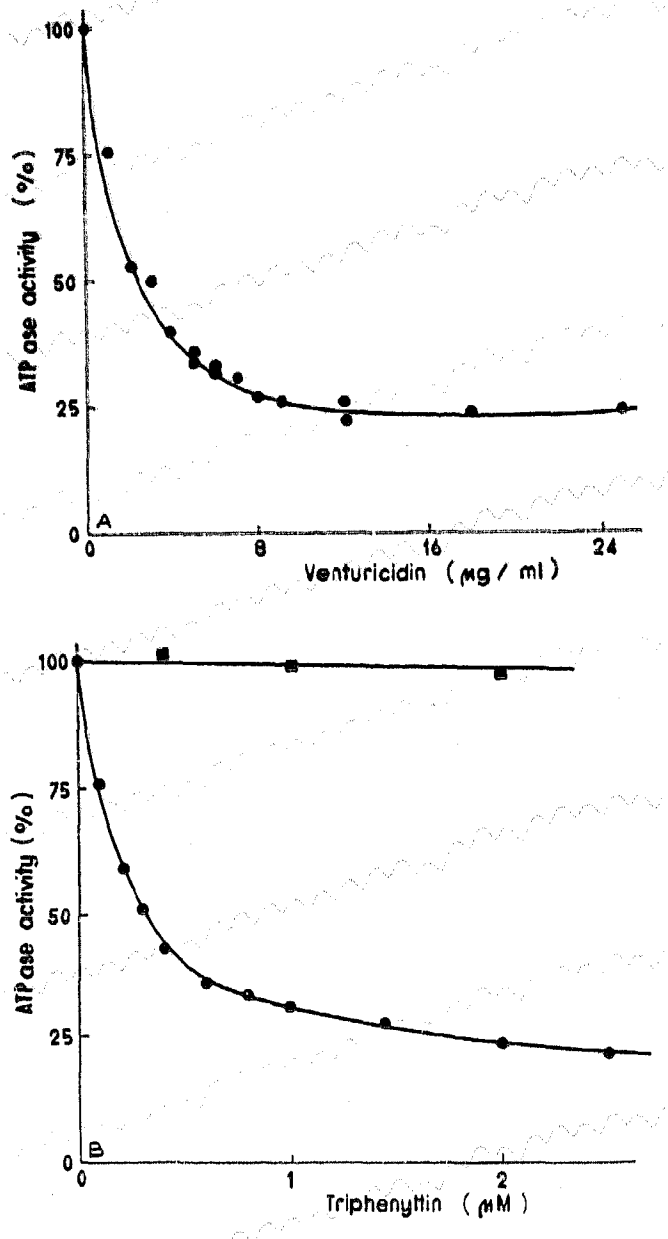

Fig. 4. Inhibition of ATPase activity of the membrane vesicles by venturicidin (A) and triphenyltin in the presence (■) and in the absence (●) of 1 mM dithiothreitol (B).

It could be interesting to compare the effect of these inhibitors on the activity of the membrane ATPase on the one hand and the ATP-dependent Na^+-transport in membrane vesicles of *V. alginolyticus*, on the other. As was found in our laboratory, (i) at low concentrations, triphenyltin and DCCD greatly enhanced the ATP-dependent Na^+-uptake in inside-out vesicles, and (ii) at higher concentrations these compounds inhibited the Na^+-transport [7]. The former effect may be caused by blocking passive Na^+-conductivity of F_o-components which have lost F_1. The latter can be attributed to inhibition of the intact F_oF_1-ATPase complex.

DCCD, venturicidin and triphenyltin were found to arrest the uncoupler-stimulated ATP-synthesis supported by an artificially imposed ΔpNa (P.A. Dibrov, V.P. Skulachev and M.V. Sokolov, unpublished data).

These data are consistent with the suggestion that Na^+-transport is mediated by an enzyme of the F_oF_1-type, probably the same that we have studied in the above experiments.

Since no other membrane ATPase was detected in *V. alginolyticus*, this F_oF_1 enzyme must be competent in the transport of either Na^+ or H^+. If such is the case, the *V. alginolyticus* ATP-synthase can utilize both the sodium and the proton potentials formed by the initial and the terminal respiratory chain segments, respectively [10]. In this respect the ATP-synthase of *V. alginolyticus* differs from that of *Propionigenium modestum* which, under native conditions, uses only the sodium potential generated by the Na^+-motive decarboxylase [11]. It seems reasonable therefore that the *P. modestum* enzyme which requires Na^+ for maximal ATPase activity is better adapted to the Na^+-motive rather than to the H^+-motive regime, whereas the *V. alginolyticus* ATPase is highly active even without Na^+.

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