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Inhibition of *Escherichia coli* H^+-ATPase by venturicidin, oligomycin and ossamycin

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The antibiotics venturicidin, oligomycin and ossamycin were investigated as potential inhibitors of the *Escherichia coli* H^+-ATPase. It was found that venturicidin strongly inhibited ATP-driven proton transport and ATP hydrolysis, while oligomycin weakly inhibited these functions. Inhibition of the H^+-ATPase by venturicidin and oligomycin was correlated with inhibition of F_0-mediate proton transport. Both inhibitors were found to interfere with the covalent reaction between dicyclohexyl[^{14}C]carbodiimide and the F_0 subunit *c* (*uncE* protein). Ossamycin had no direct inhibitory effect on *E. coli* F_0 or F_1; rather, it was found to uncouple ATP hydrolysis from proton transport.

Introduction

The H^+-ATPase (F_1F_0) of *Escherichia coli* membranes catalyses ATP synthesis and the formation of ATP-driven proton electrochemical gradients, and resembles analogous enzymes from mitochondria, chloroplasts and other bacteria [1,2]. Experiments with specific inhibitors have provided important information about the partial reactions involved in ATP synthesis and ATP-driven proton translocation. The antibiotics oligomycin, venturicidin and ossamycin inhibit the mitochondrial H^+-ATPase by interacting with the F_0-sector to block proton translocation [3]. These antibiotics are complex hydrophobic compounds whose chemical properties and structures have been well-defined [4].

Oligomycin is reported to be quite specific for H^+-ATPases from mitochondria or certain photosynthetic bacteria [4], and has been reported not to inhibit H^+-ATPases from chloroplasts [4], *E. coli* [5] or other bacteria [4]. Venturicidin and ossamycin strongly inhibit mitochondrial H^+-ATPase [3,4]; venturicidin has also been reported to inhibit H^+-ATPase from *P. denitrificans* [6]. However, the mechanism of inhibition of proton transport by these antibiotics remains obscure due, in part, to the complex and unresolved structure of the mitochondrial F_0-sector. In *E. coli*, the F_0-sector appears simpler and better characterized, consisting of only three different subunits, each of which has been sequenced [1,2]. Several authors have predicted secondary and tertiary structures of *E. coli* F_0-subunits [7–9]. Therefore, it seemed of value to investigate the action of these antibiotics on the *E. coli* enzyme.

In this report we describe for the first time the effects of venturicidin and ossamycin on the H^+-ATPase from *E. coli*, together with a reexamination of the effects of oligomycin.

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Abbreviations: DCCD, *N,N'*-dicyclohexyl[^{14}C]carbodiimide; DMSO, dimethyl sulfoxide.

Materials and Methods

Strains of E. coli used

Strain AN1460 (*unc*$^+$) was described previously [10]. Strain AN1339 (*unc*$^+$) has the genotype *unc*$^+$ *argH pyrE entA* (Gibson, F. and Cox, G.B., unpublished data).

Growth of cells, preparation of F_1-depleted membranes and purification of soluble F_1

Cells were grown to mid-log phase in 14-l batches in a New Brunswick Microferm fermentor as described by Gibson et al. [11]. The preparation of F_1-depleted membranes was performed according to Perlin et al. [12]. F_1 was purified from membranes of strain AN1460 (*unc*$^+$) by the method of Senior et al. [13] (see also Wise et al. [14]).

Measurement of ATP- and lactate-driven pH gradient formation

Fluorescence quenching of the weak base acridine orange was used to monitor pH gradient formation in membrane vesicles, as previously described [12]. A 2 ml reaction medium consisted of 20 mM Tris-HCl (pH 7.5)/200 mM KCl/5 mM $MgSO_4$/0.8 mg membrane protein/4 μM Acridine orange. Fluorescence quenching was initiated by the addition of either 2.5 mM ATP or 5 mM sodium lactate.

Potassium and valinomycin-induced pH gradient formation

F_1 depleted AN1339 (*unc*$^+$) membrane vesicles were loaded with K^+ as previously described [12], except that the equilibrating buffer consisted of 10 mM Tris-HCl (pH 7.5)/250 mM K_2SO_4/0.2 mM $MgSO_4$. The membranes were resuspended in equilibration buffer at 30 mg of protein per ml. A 10 μl aliquot of potassium-loaded vesicles (300 μg of membrane protein) was transferred to a 2 ml stirred volume (in a fluorescence cuvette) containing 20mMTris-HCl (pH 7.5), 400 mM NaCl, 5 mM $MgSO_4$, 2 μM Acridine orange and the indicated concentration of inhibitor. After 10 min,valinomycin (5 μg per mg protein) was added to generate an interior negative membrane potential. The subsequent inward proton influx resulting in pH gradient formation was monitored by the quenching of Acridine orange fluorescence.

Rate of [^{14}C]DCCD labeling of F_0 subunit c in inhibitor-treated and untreated membranes

F_1-depleted AN1460 (*unc*$^+$) membranes (10 mg of membrane protein) in 2 ml of 50 mM Tris-HCl (pH 7.5)/100 mM KCl/5 mM $MgSO_4$ were incubated with venturicidin (20 μg of inhibitor per mg of protein), oligomycin (75 μg of inhibitor per mg of protein), ossamycin (75 μg per mg of protein) or DMSO (0.5% (v/v)) for 45 min at 30°C and then cooled to 4°C. To each set of membranes, [^{14}C]DCCD was added at a final concentration of 50 μM (80000 dpm per nmol) and allowed to react at 4°C. At times 0, 1, 6, 12 and 24 h, 100-μl aliquots of each suspension were diluted with 10 ml of 50 mM Tris-HCl (pH 7.5)/100 mM KCl/5 mM $MgSO_4$, and centrifuged at 150000 × *g* for 2 h. The membrane pellets were resuspended in 0.5 ml 10 mM Tris-HCl (pH 7.5)/5 mM $MgSO_4$. Aliquots (50 μg of membrane protein) were run on SDS gels and ^{14}C-label appearing in subunit *c* was visualized by fluorography.

SDS gel electrophoresis and fluorography

SDS gel electrophoresis was performed according to Wise et al. [14]. Fluorography was performed as follows: following gel electrophoresis, SDS gels were fixed in 250 ml of 10% (v/v) acetic acid/20% (v/v) methanol for 1 h. The fixative was removed and replaced with 100 ml of AMPLIFY solution (Amersham Corporation). After incubation with continuous mixing for 1 h, the SDS gel was removed and dried down on No. 1 Whatman filter paper with a Bid-Rad gel dryer (Bid-Rad Corporation). The dried SDS gel was placed in close contact with Kodak X-OMAT AR-5 X-ray film (preflashed) in the dark at −80°C for 8 days and developed with an automated RP X-OMAT X-ray film processor (Kodak).

Assay of ATP hydrolysis

ATP hydrolysis was measured in a 1 ml reaction medium consisting of 20 mM Tris-HCl (pH 7.5)/5 mM $MgSO_4$/500 μg membrane protein. The reaction was initiated by the addition of 2.5 mM ATP and was allowed to proceed for 3 min at 30°C. It was stopped by the addition of 1 ml of 10% (w/v) SDS. Inorganic phosphate released was determined by the method of Taussky and Shorr [15].

Other procedures

The binding of purified F_1 to F_1-depleted membranes was assayed as previously described [12]. Protein content was estimated by the method of Lowry et al. [16].

Chemicals

Dicyclohexyl[^{14}C]carbodiimide (DCCD) was obtained from Amersham; Acridine orange was from Eastman Chemical Company; venturicidin was from Gallard-Schlesinger Chemical Corporation; ossamycin was the generous gift of Dr. Judith L. MacBeth, Bristol Myers Company; oligomycin (mixture containing 65% (w/w) oligomycin A, 20% (w/w) oligomycin B and 15% (w/w) oligomycin C) and all other chemicals were from Sigma. Oligomycin, venturicidin and ossamycin were dissolved in DMSO and added in assays at concentrations such that the DMSO content was less than 1% (v/v). Controls contained added DMSO alone, at the same concentration.

Results

Inhibition of ATP-driven pH gradient formation and ATP hydrolysis by venturicidin, ossamycin and oligomycin

As shown in Fig. 1A, ATP-driven pH gradient formation in AN1339 (*unc*$^+$) membrane vesicles was inhibited strongly by venturicidin and ossamycin and weakly by oligomycin as determined by the quenching of Acridine orange fluorescence. Venturicidin inhibited pH gradient formation by 50% at 9 μg of inhibitor per mg of protein (I_{50}), while ossamycin and oligomycin required 46 and 152 μg of inhibitor per mg protein, respectively. Initial rate measurements of ATP hydrolysis (made under the same conditions as in Fig. 1A) indicated that venturicidin and oligomycin inhibited ATP hydrolysis in parallel with ATP-driven proton transport (Fig. 1B); ossamycin caused little inhibition of ATP hydrolysis (Fig. 1B). There was no effect of the inhibitors on ATP hydrolysis by soluble F_1 (data not shown).

The mitochondrial H^+-ATPase appears more sensitive to inhibition by venturicidin than the *E. coli* enzyme. Reported I_{50} values for inhibition of ATPase activity by venturicidin range from 0.13 to 3.5 μg inhibitor per mg of protein for *S. cerevisiae*

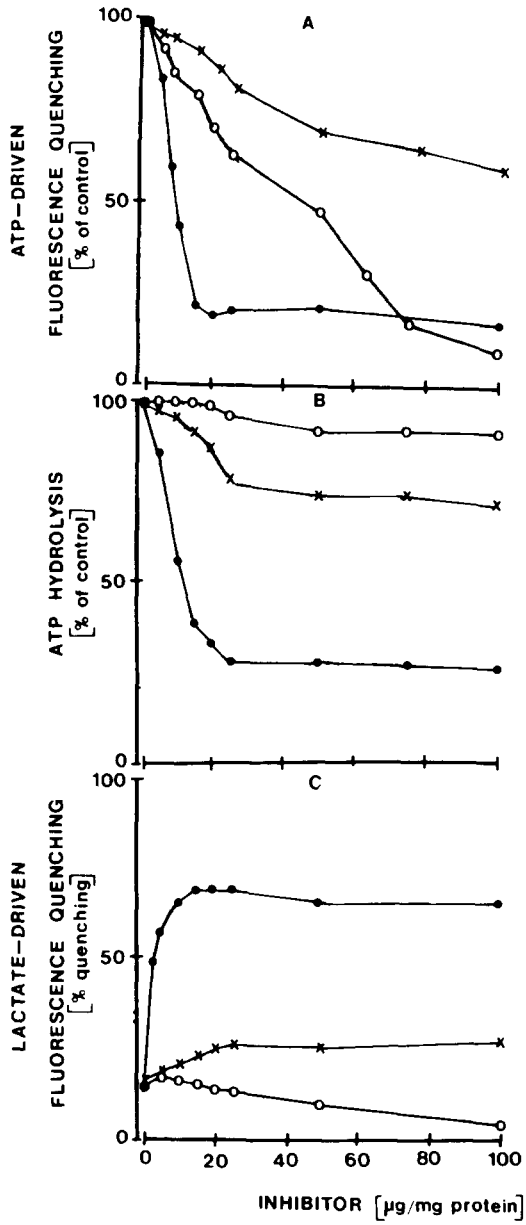

Fig. 1. Effect of venturicidin, oligomycin and ossamycin on ATP- and lactate-driven pH gradient formation and ATP hydrolysis. (A) AN1339 (*unc*$^+$) membranes were incubated with venturicidin (●——●), oligomycin (×——×) or ossamycin (○——○) at the indicated concentrations for 45 min at 30°C, and then were assayed for ATP-driven pH gradient formation as indicated by the quenching of acridine orange fluorescence (see Materials and Methods). (B) Initial rates of ATP hydrolysis were determined for membranes treated as in (A). (C) F_1-depleted AN1339 (*unc*$^+$) membranes were treated with inhibitors as in (A), and then assayed for lactate-driven pH gradient formation (see Materials and Methods). In each assay, control membranes were incubated with 0.5% (v/v) DMSO.

[17] and bovine heart [18] mitochondria, respectively, as compared to 11 μg inhibitor per mg of protein for *E. coli* membranes (Fig. 1B) *. In all cases, the inhibition by venturicidin was incomplete (i.e., did not reach 100%) (Fig. 1B and A and Walter et al. [18]).

Oligomycin inhibited mitochondrial ATPase activity by 95% at 0.4 μg inhibitor per mg protein from bovine heart [19], by 83% at 4μg inhibitor per mg protein from *Neurospora crassa* [20] and by 90% at 10 μg inhibitor per mg protein from *S. cerevisiae* [17]. By comparison, the *E. coli* H^+-ATPase was inhibited by only 28% at 100 μg of oligomycin per mg of protein (Fig. 1B), which suggests that there is only weak interaction between oligomycin and the *E. coli* proton-ATPase.

Ossamycin was a less effective inhibitor of the mitochondrial H^+-ATPase than oligomycin [6], although it inhibited bovine heart [18] and *S. cerevisiae* [17] mitochondrial ATPase activity by 90% at 10 μg of inhibitor per mg of protein. In *E. coli* membranes, ossamycin did not inhibit ATPase activity (Fig. 1B), even at concentrations that inhibited ATP-driven pH gradient formation by 90% (Fig. 1A).

Effect of venturicidin, ossamycin and oligomycin on F_0-mediated proton transport

The formation of lactate-driven pH gradients in F_1-depleted AN1339 (*unc*$^+$) membranes was assayed in order to examine the effects of inhibitors on F_0-mediated proton conduction. In this assay, an increase in the magnitude of the pH gradient (increase in percent fluorescent quenching) indicates a decrease in rate of proton transport by the F_0. Fig. 1C shows that both venturicidin and oligomycin enhanced pH gradient formation in the same concentration range that they blocked ATP hydrolysis (Fig. 1B) and ATP-driven pH gradient formation (Fig. 1A), and that venturicidin inhibited proton-conduction through F_0 strongly, whereas oligomycin did so weakly. It was also seen that when proton conduction was inhibited to the maximum extent possible with oligomycin (e.g., as in Fig. 1C), addition of venturicidin caused further inhibition to the extent normally seen with venturicidin (data not shown). Ossamycin did not inhibit F_0-mediated proton-transport, and at inhibitor levels above 25 μg per mg of protein, appeared to increase it (Fig. 1C). This latter effect was further examined in DCCD-treated AN1339 (*unc*$^+$) membranes and was found to be due to an apparent inhibition of lactate oxidation by ossamycin (data not shown).

The effects of venturicidin, ossamycin and oligomycin on F_0-mediated proton conduction were further assessed by examining proton fluxes in control and inhibitor-treated F_1-depleted membranes in response to an interior negative membrane potential generated by a K^+-gradient plus valinomycin. As Fig. 2 shows, the addition of

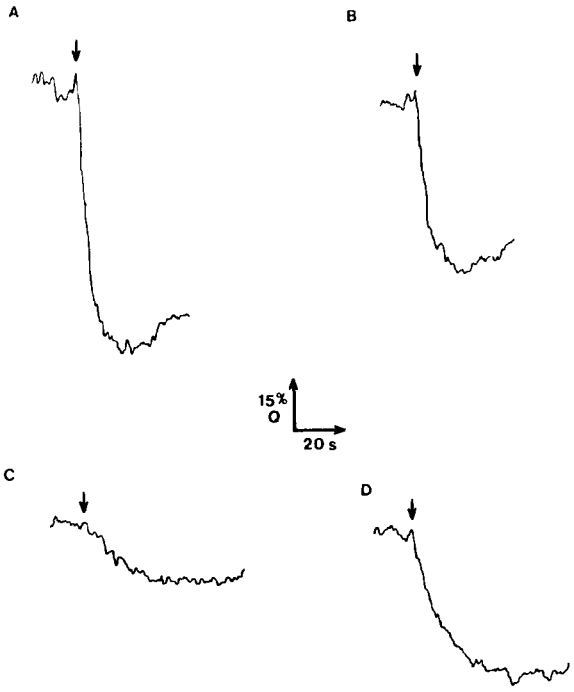

Fig. 2. Effect of venturicidin, oligomycin and ossamycin on F_0-mediated proton fluxes induced by potassium + valinomycin. Potassium-loaded, F_1-depleted AN1339 (*unc*$^+$) membrane vesicles (see Materials and Methods) (300 μg protein) in 2 ml of 20 mM Tris-HCl (pH 7.5)/200 mM KCl/5 mM $MgSO_4$/ 2 μM Acridine orange were incubated with (A) DMSO (0.5% (w/v)) (control membranes), (B) ossamycin (100 μg per mg protein), (C) venturicidin (50 μg per mg protein), (D) oligomycin (100 μg per mg protein). Valinomycin (5 μg per mg protein) was added to initiate inward proton flux through the F_0 (arrows), which was monitored by the quenching of Acridine orange fluorescence.

* It should be borne in mind that the number of nmol of H^+-ATPase per mg *E. coli* membrane protein is likely to be less than that of mitochondrial membranes.

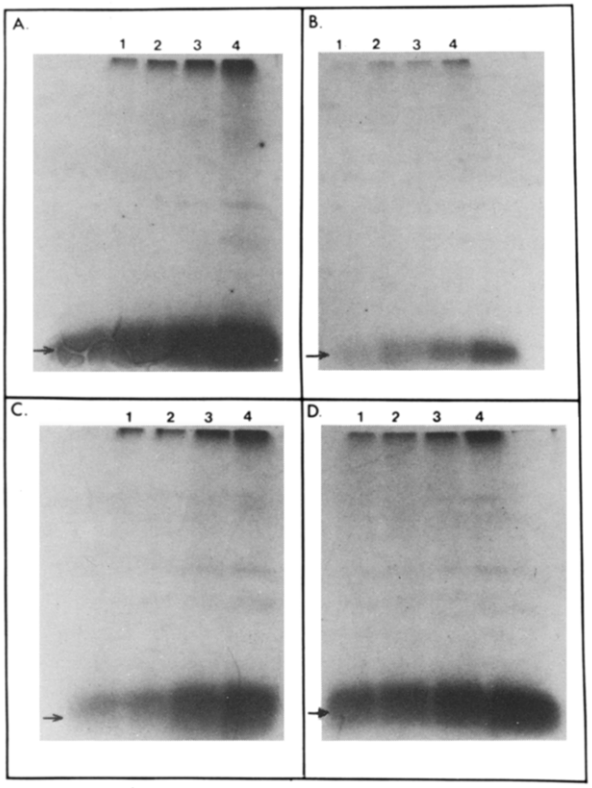

Fig. 3. Rate of [^{14}C]DCCD incorporation into subunit *c*. F_1-depleted AN1460 (*unc*$^+$) membranes were incubated with [^{14}C]DCCD (50 μM) after a pretreatment with (A) DMSO (0.5% (w/v)) (control membranes), (B) venturicidin (20 μg per mg protein), (C) oligomycin (75 μg per mg protein) or (D) ossamycin (75 μg per mg protein) for 45 min at 30°C. Aliquots (50 μg protein) of the incubation mixture were removed at 1, 6, 12 and 24 h and applied to SDS gels to resolve subunit *c*. The ^{14}C-label appearing in subunit *c* (arrows) was visualized by fluorography.

valinomycin to potassium-loaded vesicles in a low-potassium medium resulted in rapid pH gradient formation (as indicated by the quenching of acridine orange fluorescence) for control (5.1 fluorescence units per s) and ossamycin-treated membranes (4.7 fluorescence units per s). The rates were reduced by 3- to 4-fold in membranes treated with oligomycin (1.4 fluorescence units per s) and by 16- to 17-fold in membranes treated with venturicidin (0.29 fluorescence units per s) (Fig. 2). The results show that venturicidin and oligomycin inhibit proton transport through the F_0-portion of the H^+-ATPase, while ossamycin does not.

Effect of venturicidin, ossamycin and oligomycin on the rate of [^{14}C]DCCD reaction with subunit c of F_0

The fluorograms of SDS-gels in Fig. 3 show that the rate of [^{14}C]DCCD incorporation into F_0 subunit *c* (*unc*E protein) at 4°C was significantly less for membranes pretreated with venturicidin (20 μg of inhibitor per mg of protein) than for control membranes (pre-treated with 0.5% (v/v) DMSO). Membranes pretreated with oligomycin or ossamycin at 75 μg of inhibitor per mg of protein showed [^{14}C]DCCD incorporation rates that were only slightly lower than in control membranes.

Discussion

The antibiotics venturicidin, oligomycin and ossamycin were examined as potential inhibitors of *Escherichia coli* H^+-ATPase. It was found that ATP-driven proton transport and ATP hydrolysis were inhibited strongly by venturicidin and weakly by oligomycin (Fig. 1A and B) and that proton transport through the F_0 was inhibited (Figs. 1C and 2), consistent with the known effects of venturicidin and oligomycin on mitochondrial H^+-ATPase [3,4]. Ossamycin inhibited ATP-driven proton transport (Fig. 1A) but, unlike venturicidin and oligomycin, it did so by uncoupling ATP hydrolysis from proton transport. Ossamycin had no direct inhibitory effect on *E. coli* F_0 or F_1.

Because of its comparatively simple structure (see Introduction) the F_0 of *E. coli* represents a preferred system in which to study F_0-mediated proton transport. However, until now the only inhibitor known to act on the *E. coli* F_0 has been DCCD. Therefore, our finding that venturicidin potently inhibits *E. coli* F_0 proton-conduction is a new and important finding. Unlike DCCD, which also inhibits the F_1 [21], venturicidin inhibits only the F_0. The inhibition by venturicidin was found to not be reversed by diluting or washing the inhibited membranes (data not shown).

The site of venturicidin interaction with mitochondrial F_0 has been inferred previously from studies of venturicidin-resistant mutants in yeast [22,23], which showed that venturicidin-resistance was closely linked to oligomycin-resistance loci *oli1* and *oli3*. Since these loci mapped at a specific region of the mitochondrial genome shown to code

for the proteolipid subunit of yeast F_0 (analogous to subunit *c* of *E. coli* F_0) [24], it was proposed that venturicidin also interacts with this subunit [25]. Experimental support for this proposal came from studies showing that venturicidin blocked the reaction of [^{14}]DCCD with the proteolipid subunit of mitochondrial F_0 from bovine heart [26] and *N. crassa* [24]. The results presented here (Fig. 3) are in accord with the suggestion that venturicidin interacts with subunit *c* (*uncE* protein) of the *E. coli* F_0.

Clearly, it would be useful to obtain and characterize venturicidin-resistant mutants of *E. coli*. However, preliminary experiments done here using strain AN1339 (*unc*$^+$) showed that the cells grew normally in respect to both rate and growth yield with either glucose or succinate as carbon source, at venturicidin concentrations up to 100 μg per ml. Presumably, the AN1339 cells are able to detoxify the venturicidin, or to prevent it from reaching the F_0. In future studies, *E. coli* strains with defective cell walls may prove useful adjuncts in obtaining venturicidin-resistant mutants (see, e.g., Jones and Beechey et al. [27]).

Oligomycin is also presumed to interact with the proteolipid subunit of mitochondrial F_0. This idea is supported by labeling studies in which oligomycin partially blocked the incorporation of [^{14}C]DCCD into mitochondrial F_0 [24] and by genetic analyses indicating that oligomycin-resistance loci mapped in genomic regions coding for the proteolipid subunit in *N. crassa* and yeast [24]. In yeast, additional oligomycin-resistance loci (*oli2* and *oli4*) have been located on a separate gene coding for F_0-subunit 'ATPase 6' [24], indicating that oligomycin-binding involved sites on two separate F_0 subunits. Senior and Wise [1] have pointed out that although the yeast proteolipid and 'ATPase 6' subunits are strongly homologous with *E. coli* F_0 subunits *c* and *a*, respectively, one of the oligomycin-resistance loci in yeast ATPase 6 (*oli4*) is absent from subunit *a* of *E. coli*. It was proposed that the absence of this putative binding site may confer relative oligomycin insensitivity to the *E. coli* H^+-ATPase [1]. The partial inhibition of *E. coli* F_0 by oligomycin seen here (Figs. 1 and 2) is consistent with this proposal. (It should be noted that the concentration of oligomycin used in a previous report [5] was actually too low to give the partial inhibition which we observed.) Our results suggest that venturicidin and oligomycin have separate sites of action on subunit *c* of the *E. coli* F_0. A similar conclusion with regard to the mitochondrial F_0 was reached on the basis of inhibition studies on membrane-bound [18,23] and detergent-solubilized [29,30] H^+-ATPase and from genetic cross-resistance studies [22,23,25].

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