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THE EFFECT OF ASTELTOXIN AND CITREOMONTANINE, TWO POLYENIC α-PYRONE MYCOTOXINS, ON *ESCHERICHIA COLI* ADENOSINE TRIPHOSPHATASE

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ABSTRACT : Asteltoxin and citreomontanine, two polyenic α-pyrone mycotoxins closely related to aurovertins and citreoviridin, have been tested for their effect on the ATPase activity of *E. coli* BF_1. Citreomontanine was inactive towards *E. coli* ATPase. Asteltoxin inhibited *E. coli* BF_1-ATPase activity with a potency intermediate between that of citreoviridin and aurovertins. Asteltoxin had no inhibitory effect on BF_1 isolated from an aurovertin-resistant mutant. Like for aurovertin, large enhancement of the fluorescence of asteltoxin was observed upon interaction of asteltoxin with BF_1. There was no enhancement of fluorescence when citreomontanine was added to BF_1. The fluorescence response of asteltoxin was further stimulated by ADP and quenched by Mg^{2+}. The binding data showed one binding site for asteltoxin per BF_1 in the presence of ADP. No fluorescent complex was formed when asteltoxin was added to BF_1 isolated from an aurovertin-resistant mutant. In contrast to aurovertin, asteltoxin did not enhance the binding affinity of BF_1 for inorganic phosphate. Data presented in the paper indicate that, in the aurovertin family derivatives, the terminal ring system opposite to the α-pyrone end of the molecule plays a decisive role in inhibitory and binding properties with respect to ATPase.

INTRODUCTION

Aurovertin and citreoviridin have been extensively used as inhibitors of oxidative phosphorylation (1-3). Inhibition of the ATPase activity of mitochondrial F_1-ATPase (4-6) and bacterial BF_1-ATPase (7, 8) is accompanied by the binding of aurovertin to the β subunit of these ATPases and the formation of a fluorescent complex. Citreoviridin inhibits *E. coli* BF_1-ATPase, but the binding is not accompanied by an increased fluorescence.

Asteltoxin (9, 10) and citreomontanine (11), two polyenic α-pyrone compounds, have been recently isolated from *Aspergillus stellatus* and *Penicillium pedemontanum*, respectively. Their structures (1-3) shown in Fig.1, are closely related to those of aurovertin and citreoviridin. The aim of the present work, carried out with *E. coli* BF_1, was to compare the binding and inhibitory properties of asteltoxin and citreomontanine to those

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of aurovertin and citreoviridin in order to better characterize the interaction of specific groups of polyenic α-pyrone derivatives with ATPase.

MATERIALS AND METHODS

Asteltoxin and citreomontanine used for these studies were generously given by Dr. Vleggaar, CSIR, Pretoria, South Africa and Dr. S. Rebuffat, Museum Histoire Naturelle, Paris. The concentration of asteltoxin in methanol was determined from absorbance at 367 nm, using a molar absorption coefficient of 32,760 (9) and the concentration of citreomontanine was determined from absorbance at 415 nm, using a molar absorption coefficient of 57,000 (11).

BF_1-ATPases were purified from *E. coli* K12 strain AN 180 (12) and from the aurovertin-resistant mutant MA 12 (7, 8). Beef heart mitochondrial F_1 was prepared by the method of Knowles and Penefsky (13).

ATPase activity was assayed at 30°C by addition of BF_1 to a medium containing 20 mM ATP, 10 mM $MgSO_4$, 100 mM Tris-sulfate, pH 8.5 in a final volume of 0.5 ml. After a 5 min incubation, the reaction was terminated by the addition of 0.1 ml of 2.5 N perchloric acid. The phosphate released was measured by the method of Fiske and SubbaRow (14). The protein concentration of preparations of BF_1 was determined by the dye-binding procedure of Bradford (15) with bovine serum albumin as the standard. The molar amount of BF_1 was calculated assuming a molecular weight of 320,000 (16).

Fluorescence was measured at 24°C in 2 ml of 0.25 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.4, in a Perkin-Elmer MPF 2A instrument. Prior to fluorescent measurements, BF_1 was equilibrated in the above buffer by centrifugation-filtration (17) through a 1 ml column of Sephadex G50 (fine) prepared in the same medium.

Binding of P_i to BF_1 was assayed by the centrifugation-filtration technique of Penefsky (17). $^{32}P_i$ was obtained from the Commissariat à l'Energie Atomique (Saclay, France) and diluted to a specific activity of 120 cpm/picomole. BF_1 was incubated for 30 min at room temperature with 5.6 to 280 μM sodium (^{32}P)phosphate in 50 mM sucrose, 40 mM Tris, 40 mM 2-(N-morpholino)ethane sulfonic acid, 1 mM $MgSO_4$, final pH 7.4. BF_1 (10 μl, 80-100 μg protein) in 10 mM Tris-HCl, pH 7.4, was added last and the total volume was 0.1 ml. Blank samples were prepared with the protein replaced by the same volume of 10 mM Tris-HCl, pH 7.4. All solutions were prepared in freshly distilled water. At the end of the incubation period, samples were loaded on short Sephadex G50 (fine) columns which were made in 1 ml-plastic tuberculin syringes, equilibrated with the incubation buffer and inserted in 10 ml-centrifuge tubes. After centrifugation, radioactivity and protein concentration were determined on portions of the excluded eluates. The radioactivity data were corrected for blanks. The correction was less than 20% of the sample counts.

RESULTS

Asteltoxin and citreomontanine were tested for their inhibitory effects on BF_1 from wild type strain AN 180. Titration curves are shown in Fig.2A, citreomontanine, tested up to 56 μM, had no inhibitory effect. Half-inhibition was obtained at 10 μM asteltoxin. A plot of the reciprocal of asteltoxin concentration against the reciprocal of ATPase inhibition was

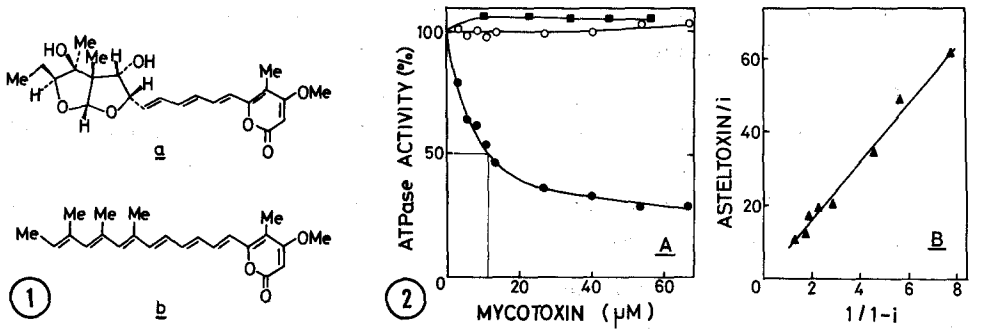

Figure 1. Chemical structures of (a) asteltoxin and (b) citreoviridin.

Figure 2. Asteltoxin titration of ATPase activity of BF_1 isolated from wild-type *E. coli* (strain AN 180) and the aurovertin-resistant mutant (strain MA 12).

A) ATP hydrolysis catalyzed by BF_1 from wild type *E. coli* AN 180 (●) and the mutant MA 12 (o) was measured in the presence of increasing concentrations of asteltoxin. Similarly ATPase activity from *E. coli* AN 180 was measured in the presence of citreomontanine (■). Methanol was kept constant (1%, v/v) in all samples. ATPase specific activities were 42 and 35 μmoles P_i released/min/mg protein for BF_1-AN 180 and BF_1-MA 12 respectively.

B) Inhibition data shown in part A for the inhibition of *E. coli* AN 180 ATPase by asteltoxin, were calculated as described in the text.

linear, by extrapolation to infinite asteltoxin concentration, a maximal inhibition of 82% was obtained (not shown). It is noteworthy that the ATPase activity of BF_1 was more than 95% sensitive to 5 mM Na-azide or 5 μM Ni-bathophenanthroline. Assuming that one molecule of asteltoxin interacts reversibly with one active site of BF_1 according to a simple equilibrium, i being the degree of inhibition for a given concentration of asteltoxin, then a plot of (asteltoxin)/i against 1/1-i should be a straight line corresponding to the equation : (asteltoxin)/i = N + K_d/1-i, where : (asteltoxin) is the concentration of asteltoxin binding sites and K_d is the apparent dissociation constant for the enzyme-inhibitor complex. Inhibition data shown in Fig.2A were recalculated for a maximal inhibition of 82% (i = 1), and plotted according to the above equation. A straight line was obtained (Fig.2B) and an apparent dissociation constant K_d = 8 μM, was measured from the slope. This affinity was not high enough for accurate determination of the number of binding sites from the intercept of the line with the ordinate axis.

The ATPase activity of BF_1 isolated from the aurovertin resistant mutant MA 12 was not inhibited by a concentration of asteltoxin as high as 60 μM, which inhibited the ATPase activity of the parent bacteria more than 75%.

Micromolar concentrations of asteltoxin in aqueous buffer were weakly fluorescent. Upon addition of BF_1, the fluorescence intensity of asteltoxin was markedly enhanced, suggesting the formation of an asteltoxin-BF_1 complex. An emission maximum was observed at 470 nm and an excitation maximum at 385 nm. A subsequent addition of ADP to BF_1-asteltoxin complex led to a further enhancement of fluorescence, the emission and excitation maxima occuring at the same wavelengths (Fig.3).

The kinetics of the changes in fluorescence intensity accompanying the interaction of asteltoxin with BF_1 and the influence of adenine nucleotides and divalent cations were monitored (Fig.4). As already mentioned, addition of BF_1 to a solution of asteltoxin resulted in a rapid fluorescence enhancement. Upon addition of ADP, an extensive and fast increase of fluorescence was observed which reached a plateau after 3 to 4 min. Further addition of ADP produced only a limited quenching of fluorescence, which was always smaller with the BF_1-asteltoxin-ADP complex than with a BF_1-aurovertin-ADP complex (8). $MgCl_2$ caused a rapid and extensive quenching, Mn^{2+} could replace Mg^{2+}. In both cases, the quenching was subsequently reversed by excess EDTA (5 mM) (not shown). Similar qualitative effects were observed with a 50 fold higher ratio of asteltoxin to BF_1.

No fluorescence enhancement was observed when citreomontanine was added to BF_1. Similarly, there was no enhancement of fluorescence when

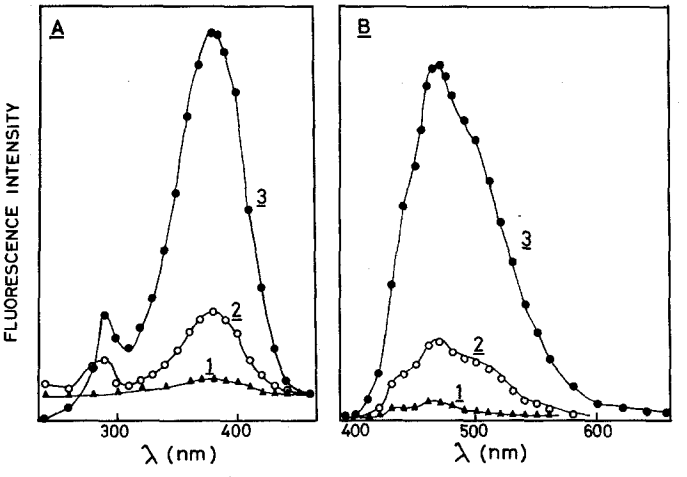

Figure 3. Fluorescence spectra of asteltoxin.

Emission spectra (A, excitation at 385 nm) and excitation spectra (B, emission at 470 nm) of 1.5 µM asteltoxin were measured as described under "Materials and Methods" (spectra 1), then in the presence of 0.2 µM of BF_1 from wild type without ADP (spectra 2) and finally with 0.5 mM ADP (spectra 3). The spectra were corrected by substracting the fluorescence of the buffer alone.

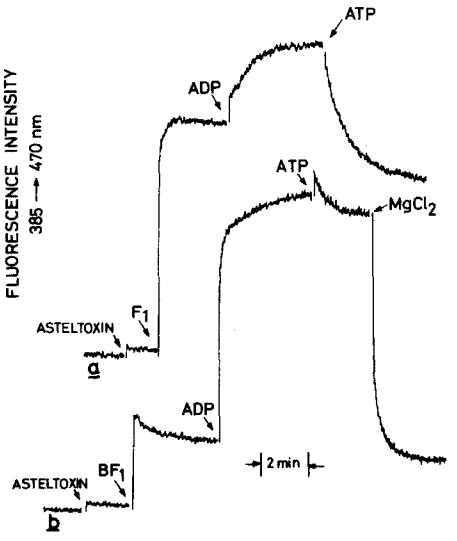

Figure 4. Effect of adenine nucleotides and Mg^{2+} on the fluorescence intensity of the BF_1-asteltoxin and F_1-asteltoxin complexes.

Measurements were performed as described in "Materials and Methods". Samples were excited at 385 nm and emission was recorded at 470 nm. Additions were made as indicated to give the following final concentrations ; 0.26 μM asteltoxin, 0.28 μM BF_1 (trace a) or 0.11 μM F_1 (trace b), 0.5 mM ADP, 2 mM ATP, and 2.5 mM $MgCl_2$.

asteltoxin was added to BF_1 isolated from the aurovertin-resistant mutant MA 12, but the presence of the mutant BF_1 did not prevent the fluorescent changes resulting from the binding of asteltoxin to wild type BF_1. Complementary experiments carried out with F_1-ATPase isolated from beef heart mitochondria also revealed the formation of an asteltoxin-F_1 complex, the fluorescence of which was stimulated by ADP and quenched by ATP (Fig.4).

Fluorescent titration of wild-type BF_1 with asteltoxin together with Scatchard plot (18) representations of the data are shown in the Fig.5. The binding ratio of asteltoxin to BF_1 were calculated as described by Chang and Penefsky (4) for aurovertin. In the presence of ADP, it was found that each mole of BF_1 bound 0.82 mole of asteltoxin with a dissociation constant (K_d) of 0.3 μM for the asteltoxin-BF_1-ADP complex. In the absence of ADP, BF_1 bound only 0.2 mole of asteltoxin (K_d = 0.1 μM). These data suggest that each BF_1, in the presence of ADP, binds one molecule of asteltoxin.

Inorganic phosphate, in the presence of Mg^{2+}, was bound by *E. coli* BF_1. The action of asteltoxin was investigated and compared to the behavior of aurovertin D. Double reciprocal plots of binding data shown in the Fig.6, indicated in all cases binding stoichiometries of about one binding site for P_i. The dissociation constants : 40 μM for the control, 55 μM in the presence of asteltoxin and 12 μM in the presence of aurovertin D, were

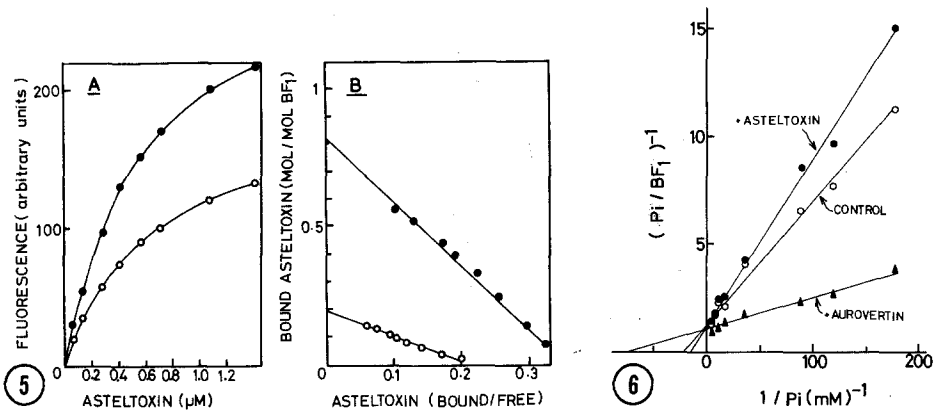

Figure 5. Fluorescence titration of BF_1.

(A) 0.53 μM BF_1 (o) and 0.23 μM BF_1 plus 0.5 mM ADP (•) were titrated with asteltoxin. Fluorescence intensities of the BF_1-asteltoxin complexes were corrected for the fluorescence of asteltoxin alone. (B) The titration data (part A) were plotted according Scatchard (18). In these experiments, the fluorescence increment for 0.1 μM asteltoxin at infinite protein concentration was 170 units.

Figure 6. Binding of P_i by BF_1.

The reciprocal of the molar binding ratio $(P_i/BF_1)^{-1}$ is plotted versus the reciprocal of the P_i concentration. Experimental procedure is detailed under "Material and Methods". Incubation mixture were supplemented with 10 μM aurovertin D (▲) or 100 μM asteltoxin (•), added as methanolic solutions. Controls (o) contained an equivalent amount of methanol (1%, v/v).

calculated from the reciprocal of the intercepts of the lines with the abcissa axis. Thus aurovertin increased the binding affinity of P_i for BF_1, in contrast, the titration in the presence of asteltoxin was similar to the control curve with even a slightly decreased affinity. The stimulation of phosphate binding by aurovertin D was demonstrated earlier for beef heart mitochondrial F_1 (19).

DISCUSSION

The data reported in this paper show that asteltoxin, but not citreomontanine, inhibits wild type *E. coli* BF_1 ATPase activity. Asteltoxin has no effect on ATPase isolated from an aurovertin-resistant mutant strain MA 12, also resistant to citreoviridin. It is noteworthy that ATPase from the *E. coli* mutant MA 12, resistant to aurovertin, citreoviridin and asteltoxin was sensitive to other ATPase inhibitors, namely dicyclohexylcarbodiimide, N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline, 4-chloro-7-nitrobenzofurazan, and azide (7). The affinity of BF_1-ATPase

for asteltoxin, K_d = 8 μM, calculated from inhibition data, is intermediate between that for aurovertin D (K_d = 0.9 μM) and that for citreoviridin (K_d = 60 μM) (8).

It has been shown that aurovertin binds to the β subunit of *E. coli* BF_1 (8, 20), as it does for mitochondrial F_1 (21, 22). The site of action of asteltoxin is likely to be identical to that of aurovertin and probably also to that of citreoviridin. This could be expected, since the structure of asteltoxin is closely related to that of aurovertin and citreoviridin (2, 10, 11). In fact, all compounds possess an α-pyrone linked to a polyenic chain, they essentially differ by their opposite terminal ring system which may explain the different degrees of affinity of BF_1-ATPase for the inhibitors. Citreomontanine with its simpler structure was no longer recognized by *E. coli* BF_1. In addition to aurovertins, asteltoxin appears to be a valuable fluorescent probe of *E. coli* BF_1 and mitochondrial F_1-ATPase as detailed in this paper. The enhancement of fluorescence intensity observed when asteltoxin is bound to BF_1, as well as the stimulating effect of ADP and the quenching effect of Mg^{2+}, are similar to the behavior of aurovertin D. However, one difference is the smaller quenching effect of ATP. Another decisive difference between aurovertin and asteltoxin is the absence of stimulatory effect of asteltoxin on phosphate binding by BF_1.

Finally, it is interesting to note the partial site reactivity of asteltoxin ; in spite of the fact that BF_1 possesses 2 or 3 β subunits, the binding of asteltoxin to one of them leads to full inhibition.

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