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Interaction of *Escherichia coli* Adenosine Triphosphatase with Aurovertin and Citreoviridin: Inhibition and Fluorescence Studies

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Aurovertins B and D inhibited the adenosine triphosphatase (ATPase) activity of soluble *Escherichia coli* coupling factor ATPase (BF_1) isolated from wild-type *E. coli* K-12. Half inhibition was obtained with 2 μM aurovertin B and 0.9 μM aurovertin D. Aurovertins B and D had no inhibitory effect on BF_1 isolated from the aurovertin-resistant *E. coli* mutant MA12. Acetylation or saponification of aurovertin D yielded a derivative which was devoid of inhibitory effect on BF_1. Citreoviridin also inhibited wild-type BF_1 but with much less efficiency (half inhibition at 60 μM) than aurovertin. Citreoviridin had no effect on the aurovertin-resistant BF_1. The fluorescence intensity of aurovertins B and D was markedly enhanced upon addition to purified BF_1. There was no enhancement of fluorescence when the aurovertins were added to BF_1 isolated from the aurovertin-resistant mutant. The fluorescence of the aurovertin-BF_1 complex was enhanced by adenosine 5'-diphosphate and by low concentrations of adenosine 5'-triphosphate. The adenosine 5'-diphosphate-enhanced fluorescence of the aurovertin-BF_1 complex was quenched by high concentrations of adenosine 5'-triphosphate or by Mg^{2+}. Aurovertin bound selectively to the β subunit of BF_1 isolated from wild-type cells. By complementation assays in vitro, using a reconstituted system made of subunits isolated from wild-type and aurovertin-resistant BF_1, it was shown that the altered peptide in aurovertin-resistant BF_1 was the β subunit.

Aurovertins and the related compound citreoviridin (Fig. 1) are potent and specific inhibitors of mitochondrial coupling factor ATPase (F_1) (4, 16, 21, 23, 25, 31). Interaction of aurovertins B and D with F_1 results in strong enhancement of their fluorescence intensity; this fluorescence enhancement is sensitive to ligands of F_1 such as ADP, ATP, phosphate, and magnesium ions (5, 9, 10, 22, 28, 39-41). It has been shown that aurovertin binds to the β subunit of F_1 in beef heart (28, 41) and in *Saccharomyces cerevisiae* mitochondria (12). *S. cerevisiae* mutants with a mitochondrial ATPase resistant to aurovertin have been described (13).

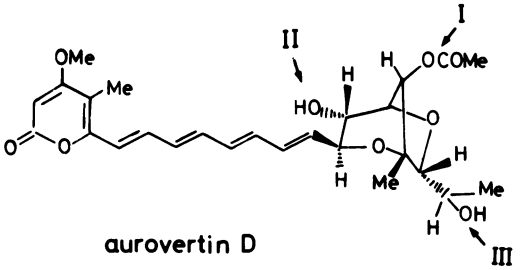
Several bacteria including *Alcaligenes faecalis* (1), *Escherichia coli* (35, 37), *Paracoccus denitrificans* (19), *Rhodospirillum rubrum* (18, 30, 38), *Rhodopseudomonas capsulata* (26), and *Rhodopseudomonas sphaeroides* (34) have been reported to be sensitive to aurovertin D, but no detailed investigation on the interaction of aurovertin with ATPase has been reported. *E. coli* mutants insensitive to aurovertin have been isolated in this laboratory (35). They possess an aurovertin-resistant ATPase, which remains sensitive to a number of other ATPase inhibitors. The aurovertin mutation maps in the *unc* region of the bacterial chromosome like other mutations of the ATPase complex (7, 8, 14).

This paper deals with the interaction of aurovertins B and D and chemically related derivatives with BF_1 isolated from wild-type *E. coli* and from an aurovertin-resistant mutant, MA12. In contrast to Van de Stadt et al. (40), we observed strong enhancement of aurovertin fluorescence upon addition of aurovertin to wild-type BF_1 but not to BF_1 isolated from the aurovertin mutant. The BF_1 subunit which possesses the aurovertin site was identified with the β subunit. Identification assays were based on the ability of the isolated β subunit from the wild-type strain to enhance the fluorescence of aurovertin and also on the resistance to aurovertin of the hybrid enzyme obtained by association of α, γ, and ϵ subunits from the wild-type strain and the β subunit from mutant MA12.

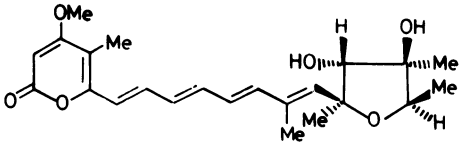
MATERIALS AND METHODS

Citreoviridin was obtained from Cambrian Chemicals (Suffolk House, Croydon, U.K.). A molar absorption coefficient of 48,000 at 383 nm (33) was used to determine the concentration of methanolic solutions.

Preparation of aurovertins. Aurovertins B and



aurovertin D



citreoviridin

FIG. 1. *Structures of aurovertin D and citreoviridin (23). Me = CH_3.*

D (Fig. 1) were extracted and purified from the mycelium and growth medium of 3-week-old cultures of *Calcarisporium arbuscula* (NRRL 3705) as described by Osselton et al. (29). Methanolic solutions protected from light were kept at −20°C, and their concentrations were determined by using a molar absorption coefficient at 34,400 at 372 nm for aurovertin B (27).

The UV absorption spectrum of aurovertin D in methanol showed peaks at 368 nm (ϵ = 35,100) and at 275 and 270 nm (ϵ = 30,000). The parent peak in the mass spectrum, M^+ = 476.202, is consistent with the formula $C_{25}H_{32}O_9$. The infrared absorption spectrum in KBr included main bands at 3,440, 1,740, 1,690, 1,625, 1,540, 1,455, 1,410, 1,375, 1,255, 1,230, 1,095, and 1,035 cm^{-1}. The above-described properties of our aurovertin D preparation are in agreement with the data published previously (2) (R. M. Bertina, Ph.D thesis, University of Amsterdam, Amsterdam, The Netherlands, 1972).

Chemical modification of aurovertin D. Aurovertin D was diacetylated on the OH groups at positions II and III (Fig. 1) by overnight incubation in the dark at room temperature in a mixture of acetic anhydride and pyridine (1:5, vol/vol). The mass spectrum of the diacetylated derivative gave an M^+ value of 560. Its infrared spectrum retained all absorption bands on aurovertin D, with a marked increase at 1,230 and 1,750 cm^{-1}, consistent with the gain of two acetate groups. Aurovertin D was saponified by incubation at 37°C for 20 min with 0.1 N NaOH in methanol. The infrared spectrum of saponified aurovertin differed from that of aurovertin by the disappearance of bands at 1,235 and 1,740 cm^{-1}, which was consistent with the loss of the acetate group at position I in the aurovertin molecule (Fig. 1). The UV spectra of the two derivatives were identical to that of aurovertin D. Their concentrations were determined assuming the same molar extinction coefficient as that of aurovertin D (ϵ = 35,100 at 368 nm).

Fluorescence measurements. Aurovertin fluorescence was measured with a Perkin Elmer MPF-2A fluorimeter. The excitation wavelength was set at 365 nm, and the emission wavelength was at 470 nm.

Bacteria and growth conditions. The wild-type BF_1 was prepared from *E. coli* K-12 AN180 (*argE3 thi-1*) (7). The original aurovertin-resistant mutants had been obtained after treatment of the parent strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (35). To eliminate any undesirable mutations possibly occurring outside of the *unc* region of the *E. coli* chromosome, a strain isogenic to parent AN180 was prepared by P1-mediated transduction. A phage P1 lysate obtained on mutant MA1 was used to infect strain AN120 (*argE3 thi-1 uncA401*) (7). Transductants were selected on minimal agar plates supplemented with arginine, thiamine, succinate, acetate, and malate as carbon sources. Strain MA12 was chosen among the recombinants capable of growth; it contained an ATPase activity totally resistant to 20 μM aurovertin D.

Bacteria were grown on rich medium (1% tryptone [Difco Laboratories], 0.5% yeast extract, and 1% NaCl) and stored frozen at −80°C before use.

BF_1 ATPase purification. Membranes were prepared by disruption of *E. coli* cells in a Sorvall-Ribi press (32). BF_1 was released from the membranes by chloroform treatment (3). It was purified by chromatography on a DEAE-cellulose column (DE 52 Whatman) at 0 to 4°C, using a buffer containing a Tris-hydrochloride gradient from 0.05 to 0.8 M, 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP (pH 7.4), and 20% (vol/vol) methanol (42). Fractions containing the ATPase activity were eluted at approximately 0.2 M Tris-hydrochloride. They were pooled, concentrated by filtration on Amicon XM-100 membranes, and further purified by gel filtration on a Sepharose 6B column equilibrated with the above-mentioned buffer. Purified BF_1 (3 to 10 mg of protein per ml) was stored at 0 to 4°C.

Dissociation of BF_1 and separation of subunits. The procedure of Vogel and Steinhart (42) was followed for dissociation of BF_1 and subunit separation. All operations were carried out at 2 to 4°C. BF_1 (1.6 mg) was dialyzed for 2 h against 50 mM morpholine ethanesulfonate buffer (pH 6.1) containing 5 mM ATP and 1 M LiCl, and then frozen and thawed twice. The protein fraction was desalted by dialysis against 50 mM morpholine propanesulfonate buffer (pH 6.5) containing 2.5 mM ATP and 2.5 mM 2-mercaptoethanol, and applied to a DEAE-cellulose column (1.2 by 4 cm, Whatman DE 52) equilibrated with the above-mentioned buffer. The column was eluted with a linear gradient of 0 to 0.5 M LiCl in the equilibration buffer.

ATPase assay. Mg ATPase activity was assayed at 37°C as described by Butlin et al. (8). The reaction mixture contained 20 mM ATP, 10 mM $MgSO_4$, and 100 mM Tris-sulfate buffer (pH 8.5) in a final volume of 0.5 ml. After a 10-min incubation, the reaction was terminated by the addition of 0.1 ml of 2.5 N perchloric acid, and the amount of phosphate released was measured colorimetrically (17). Under these conditions, the specific activity of purified BF_1 varied between 30 and 40 μmol/min per mg of protein.

Protein determination. The protein concentra-

tion was determined with the Folin-Ciocalteu phenol reagent, using bovine serum albumin as a standard (43).

RESULTS

Inhibitory effect of aurovertins B and D on BF_1 from wild-type cells and mutant MA12. Aurovertins B and D inhibited the ATPase activity of purified BF_1 from wild-type *E. coli*. Full inhibition was not attained even at high inhibitor concentrations. Inhibition was only 60% at 20 μM aurovertin B and 84% at 20 μM aurovertin D. The apparent dissociation constants (K_d) of the aurovertin-BF_1 complexes, calculated as described by Linnett et al. (24), were 0.9 μM for aurovertin D and 2.0 μM for aurovertin B (Fig. 3A).

The ATPase activity of BF_1 isolated from the aurovertin-resistant mutant MA12 was not inhibited by 20 μM aurovertin D (Fig. 2A). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing in urea-polyacrylamide gel electrophoresis and isoelectric focusing in urea-polyacrylamide gels failed to reveal detectable differences between BF_1 purified from wild-type and MA12 mutant cells. It must be noted that our ATPase preparation almost completely lacked the δ subunit, a feature possibly related to use of chloroform to release BF_1 from the bacterial membrane (11).

Inhibition of BF_1 ATPase activity by citreoviridin. A typical titration curve of BF_1 ATPase activity by citreoviridin is shown in Fig. 4. As already discussed for aurovertin, complete inhibition of ATPase activity by citreoviridin could not be achieved. The maximal inhibition (calculated for an infinite concentration of citreoviridin) was 80%, and the K_d value, calculated by the method of Linnett et al. (24), was 60 μM (Fig. 3B).

The aurovertin-resistant MA12 mutant was also resistant to citreoviridin. Citreoviridin at a final concentration of 180 μM, which inhibited the ATPase activity of the parent strain to an extent of 62%, had no effect on the ATPase activity of this mutant (Fig. 4).

pH dependence of inhibition of BF_1 ATPase by aurovertin or citreoviridin. The op-

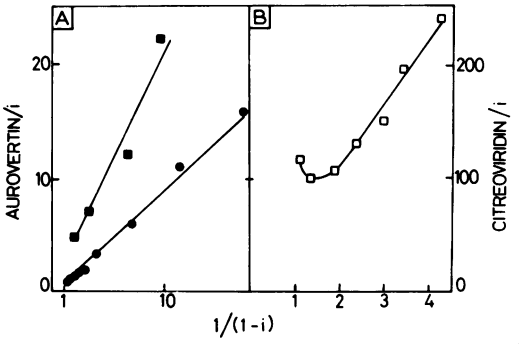


FIG. 3. *BF_1 ATPase inhibition by aurovertins and citreoviridin. The inhibition data shown in Fig. 2 for aurovertin B (■) and aurovertin D (●) and in Fig. 4 for citreoviridin (□) were plotted by the method of Linnett et al. (25). The degree of inhibition (i) was calculated for maximal inhibitions of 68, 86, and 80% for aurovertin B, D, and citreoviridin, respectively.*

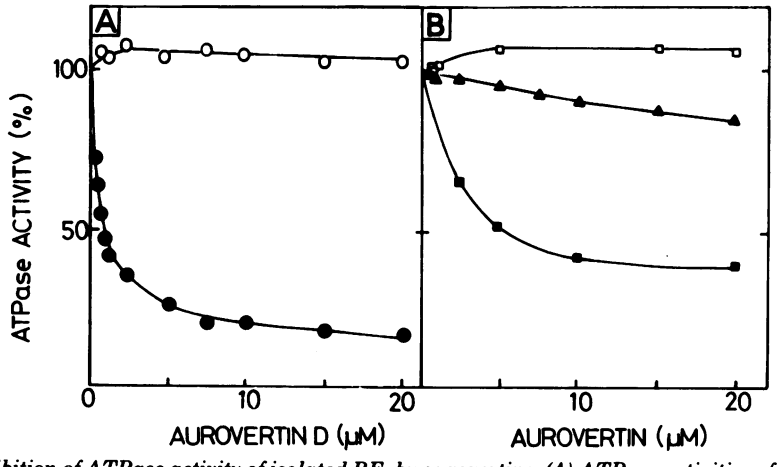


FIG. 2. *Inhibition of ATPase activity of isolated BF_1 by aurovertins. (A) ATPase activities of BF_1 extracted from strain AN180 (2.7 μg of protein, ●) and strain MA12 (3.7 μg of protein, ○) were measured in the presence of increasing concentrations of aurovertin D. (B) ATPase activities of BF_1 extracted from strain AN180 (2.7 μg of protein) were measured in the presence of increasing concentrations of aurovertin B (■), saponified aurovertin D (▲), and acetylated aurovertin D (□). Incubation conditions were as described for the ATPase assay in Materials and Methods. The methanol concentration was kept constant (1%, vol/vol) in all samples.*

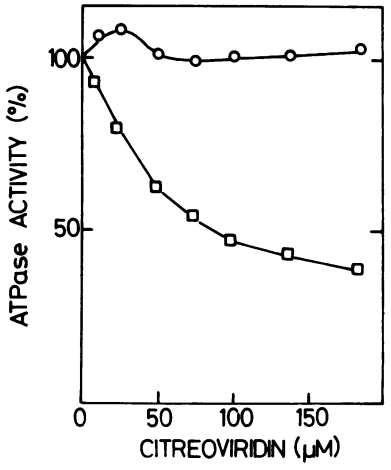


FIG. 4. *Inhibition of ATPase activity of isolated BF_1 by citreoviridin. ATPase activity of BF_1 extracted from strain AN180 (2.6 μg of protein, □) or strain MA12 (3.7 μg of protein, ○) was measured in the presence of increasing concentrations of citreoviridin.*

timal pH for the control assay, without added inhibitors, ranged between pH 8 and pH 9 (see also references 20 and 32). Maximal inhibition of ATPase activity was around pH 8.5 for aurovertin and around pH 7.5 for citreoviridin.

Changes of aurovertin fluorescence upon addition of BF_1: effect of adenine nucleotides and magnesium. Addition of aurovertin B or D to BF_1 from wild-type cells resulted in a 10- to 15-fold enhancement of the fluorescence intensity of aurovertin (Fig. 5). ADP further increased the fluorescence of the aurovertin-BF_1 complex, which reached a threefold-higher plateau after 3 to 4 min. ATP and Mg^{2+} then partially quenched the fluorescent response (Fig. 5). Quenching by Mg^{2+} did not require the presence of ATP and was reversed by an excess of EDTA. Mg^{2+} could be qualitatively replaced by other divalent cations such as Mn^{2+}, Ca^{2+}, Cd^{2+}, Ni^{2+}, and Co^{2+} but not by Ba^{2+} or Zn^{2+}.

The effect of ADP and ATP on the fluorescence of the aurovertin-BF_1 complex was inves-

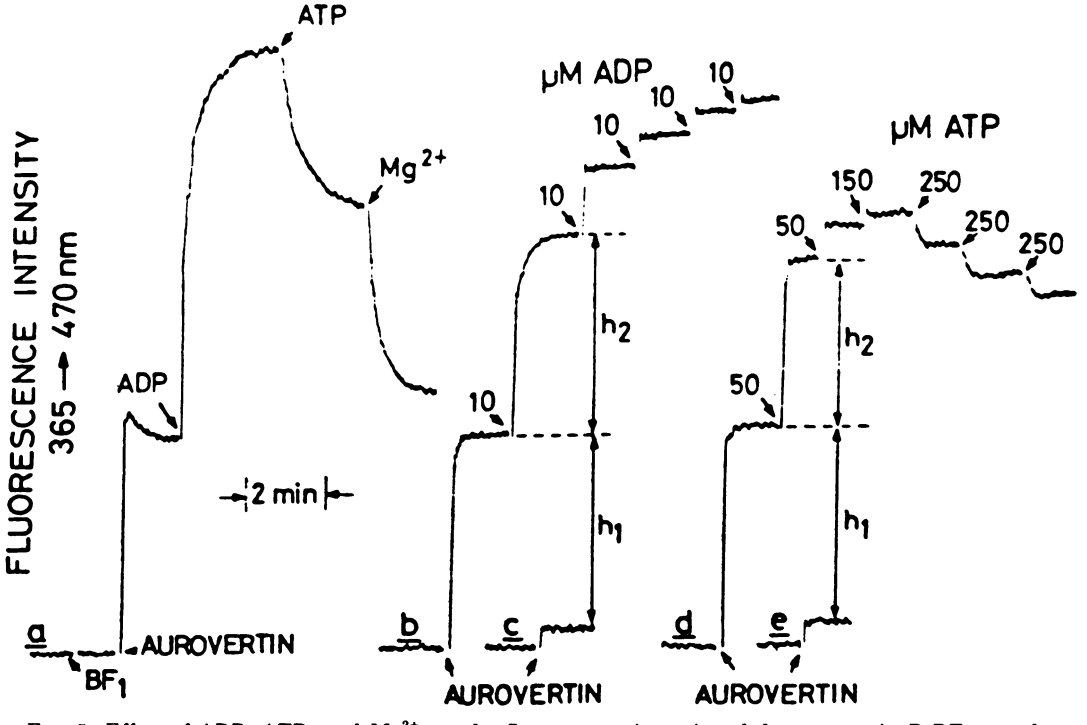


FIG. 5. *Effect of ADP, ATP, and Mg^{2+} on the fluorescence intensity of the aurovertin D-BF_1 complex. Fluorescence measurements were made at 25°C in 2 ml of 0.25 M sucrose–10 mM Tris-hydrochloride–0.5 mM EDTA (pH 7.4) with 0.4 μM aurovertin D and 0.1 mg of BF_1 from strain AN180. In trace (a), ADP, ATP, and $MgSO_4$ were added sequentially to obtain the following final concentrations: 0.2 mM ADP, 2 mM ATP, and 2.5 mM $MgSO_4$. The fluorescence of 0.4 μM aurovertin D in the above medium without BF_1 is shown in traces (c) and (e). Additions of ADP [trace (b)] or ATP [trace (d)] were made as indicated. The fluorescent intensity of the BF_1-aurovertin D complex (h_1) was obtained by subtracting the contribution of aurovertin D from the total fluorescence; and h_2 was the nucleotide-induced increment of fluorescence.*

tigated by using fixed concentrations of BF_1 and aurovertin (Fig. 5). The basal fluorescence of the aurovertin-BF_1 complex rapidly increased upon addition of ADP, the half-maximal increase being obtained at 10 to 20 μM ADP. The best fitted curve (Fig. 6) that accounts for the ADP-induced enhancement of fluorescence of the aurovertin-BF_1 complex is described by the equation $\phi = \phi_{max} \times \{[ADP]/([ADP] + K_d)\}$, where ϕ is the fluorescence enhancement ($\phi = h_2/h_1$, Fig. 5) for a given concentration of ADP, and ϕ_{max} is the maximal enhancement observed with saturating concentrations of ADP. This equation is that of a single site and gave a K_d value for ADP of 10 to 20 μM.

ATP also stimulated the basal fluorescence of the aurovertin-BF_1 complex, but high concentrations of ATP were less effective than low concentrations. The fluorescence was first enhanced by ATP concentrations up to 100 μM; then a plateau was attained for ATP concentrations ranging between 100 and 300 μM. Further additions of ATP resulted in a decrease of fluorescence. This biphasic effect can be accounted for by a dual-site model expressed by the equation $\phi = \phi_{max}\{[ATP]/([ATP] + K_1)\} - \phi_{max}\{[ATP]/([ATP] + K_2)\}$, where ϕ is the fluorescence enhancement for a given concentration of ATP, and ϕ_{max} is the maximal fluorescence enhancement attained with saturating concentration of ADP (Fig. 5). A high-affinity site of ATP (K_1 = 50 to 60 μM) would therefore be responsible for fluorescence enhancement, and a low-affinity ATP site (K_2 = 500 to 600 μM) would be responsible for fluorescence quenching. ADP might bind only to the high-affinity site (K_d = 10 to 20 μM), resulting only in fluorescence enhancement.

Starting from the high level of fluorescence obtained in the presence of saturating concentrations of ADP, the fluorescence intensity of the aurovertin-BF_1 complex was quenched upon addition of ATP (Fig. 5). This quenching effect was characterized by a K_d for ATP of 500 to 600 μM, which is identical to that calculated for the low-affinity ATP site in the dual-site model described above.

Aurovertin fluorescence increased hyperbolically with increasing concentrations of aurovertin, yielding a linear double-reciprocal plot. The apparent affinity (K_d) of aurovertin for BF_1 was 0.7 μM, close to the value deduced from inhibition experiments. ADP increased both the apparent affinity of aurovertin for BF_1 (K_d = 0.3 μM) and the maximal extent of fluorescence (Fig. 7). No enhancement of fluorescence was observed by addition of aurovertins B and D to BF_1 isolated from the aurovertin-resistant MA12 mutant whose ATPase activity is insensitive to aurovertin (35, 37).

Acetylated or saponified derivatives of aurovertin D, which do not inhibit the ATPase activity of BF_1, exhibited only a small enhancement

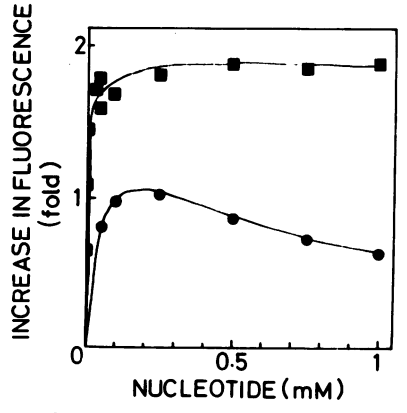


FIG. 6. *Stimulation of the fluorescence of the aurovertin-BF_1 complex by adenine nucleotides. The nucleotide-induced enhancement of fluorescence over the basal fluorescence of the aurovertin-BF_1 complex ($\phi = h_2/h_1$; Fig. 5) was plotted against the concentrations of added ADP (■) or ATP (●). Solid lines drawn through the experimental points were calculated as described in the text.*

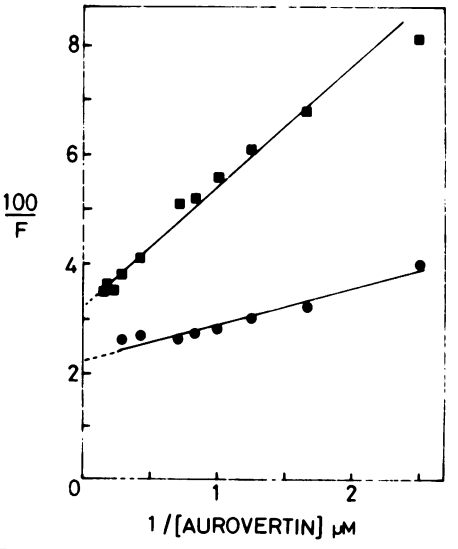


FIG. 7. *Double-reciprocal plots of the fluorescence enhancement of aurovertin D in the presence of 0.2 mg of BF_1 from strain AN180. The fluorescence was measured at 25°C with increasing concentrations of aurovertin D in 0.25 M sucrose–10 mM Tris-hydrochloride–0.5 mM EDTA (pH 7.4) (■) and supplemented with 0.2 mM ADP (●). Fluorescent intensities (F) were corrected by subtracting the fluorescence of aurovertin D alone (Fig. 5). 100/F is given in arbitrary units.*

of fluorescence (1.6- to 1.8-fold) when added to AN180 BF_1. These derivatives, similar to aurovertin D (9, 22), showed a higher fluorescence intensity in glycerol at 0°C than at 25°C.

Analysis of the defect in BF_1 from aurovertin-resistant mutant MA12. Subunits of BF_1 purified from wild-type strain AN180 were isolated by DEAE-cellulose chromatography (42) (Fig. 8). Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 9). The first protein peak eluted with 50 mM LiCl contained the α, γ, and ϵ subunits. The second peak eluted with 100 mM LiCl contained the pure β subunit. Binding of aurovertin to the different subunits was assessed by the increase in fluorescence intensity. Fluorescence enhancement was observed with protein fractions corresponding to the β subunit, indicating that aurovertin binds specifically to this subunit in BF_1. There was only a small increase in fluorescence upon further addition of ADP and no quenching effect of ATP or Mg^{2+}. These data taken together suggest that isolated β subunit from wild-strain BF_1 is still able to recognize aurovertin; however, the interactions between the aurovertin-binding site and the ADP, ATP, or Mg^{2+} site, which are typical of undissociated BF_1, are markedly weakened or even lost in the isolated β subunit. In contrast with the marked enhancement of fluorescence caused by addition of aurovertin to the β subunit isolated from wild-strain BF_1, subunit β isolated from BF_1 purified from aurovertin-resistant cells did not enhance aurovertin fluorescence, which indicates that this subunit had lost affinity for aurovertin.

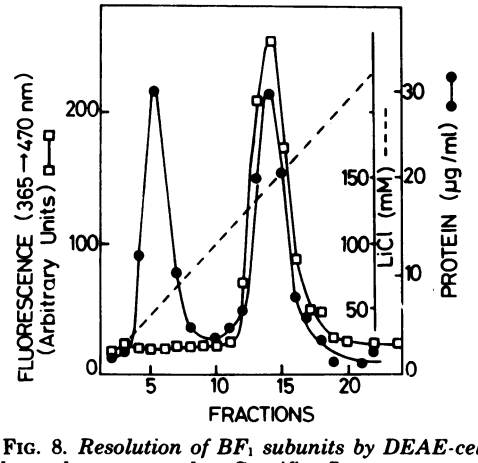


FIG. 8. *Resolution of BF_1 subunits by DEAE-cellulose chromatography. Specific fluorescence enhancement of aurovertin bound to subunit β. Details are described in the text. Fractions of 2 ml were collected and monitored for their protein content (●) and fluorescence at 470 nm (excitation at 365 nm) after addition of 0.2 μM aurovertin D (□).*

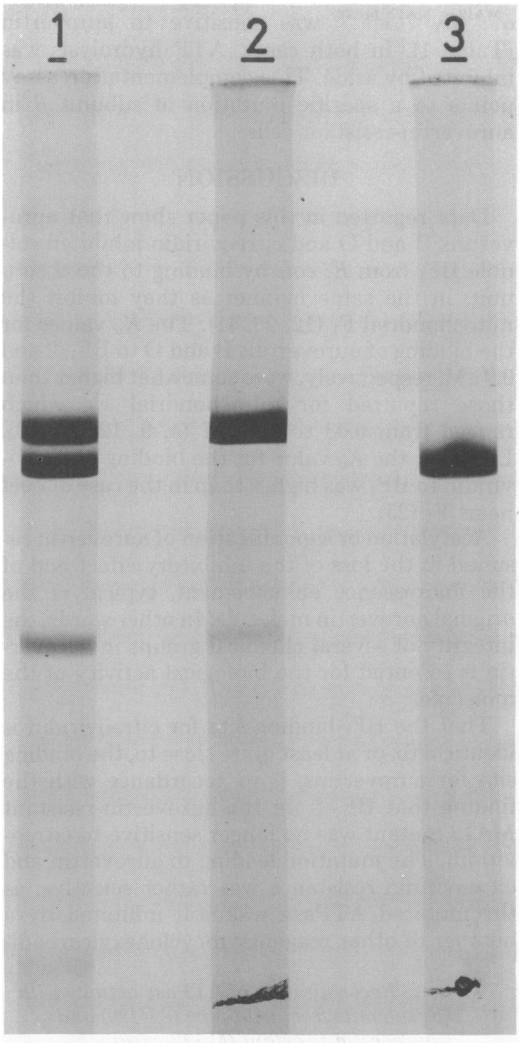


FIG. 9. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of BF_1 ATPase and its subunits. BF_1 (20 μg of protein, gel 1), the αγε fraction (10 μg of protein, gel 2), and the β fraction (10 μg of protein, gel 3) were treated with 1% sodium dodecyl sulfate (wt/vol) and 1% (wt/vol) 2-mercaptoethanol and subjected to electrophoresis on 7.5% polyacrylamide gels run in 25 mM Tris–0.19 M glycine buffer (pH 8.4) containing 0.1% sodium dodecyl sulfate (wt/vol). The position of the bromophenol blue was marked with India ink. Protein bands were detected by staining with Coomassie brilliant blue.*

The defective subunit in BF_1 from the aurovertin-resistant mutant was also identified by a complementation assay with isolated subunits. The ATPase activity was reconstituted by combining subunits from wild-type and mutant BF_1. BF_1 made of $\alpha\beta^{MA12}\gamma\epsilon$ was resistant to inhibition by aurovertin; in contrast, BF_1 made of

$\alpha^{MA12}\beta\gamma^{MA12}\epsilon^{MA12}$ was sensitive to aurovertin (Table 1). In both cases, ATP hydrolysis was inhibited by azide. This complementation assay points to a specific mutation of subunit β in aurovertin-resistant cells.

DISCUSSION

Data reported in this paper show that aurovertins B and D and citreoviridin inhibited soluble BF_1 from *E. coli* by binding to the β subunit, in the same manner as they inhibit the mitochondrial F_1 (12, 28, 41). The K_d values for the binding of aurovertins B and D to BF_1, 2 and 0.9 μM, respectively, were somewhat higher than those reported for mitochondrial F_1, which ranged from 0.03 to 1.4 μM (5, 9, 12, 28, 40). Likewise, the K_d value for the binding of citreoviridin to BF_1 was higher than in the case of beef heart F_1 (25).

Acetylation or saponification of aurovertin resulted in the loss of the inhibitory effect and of the fluorescence enhancement, typical of the original aurovertin molecule. In other words, the integrity of several chemical groups in aurovertin is essential for the biological activity of the molecule.

That the BF_1-binding site for citreoviridin is identical to, or at least quite close to, the binding site for aurovertins, is in accordance with the finding that BF_1 from the aurovertin-resistant MA12 mutant was no longer sensitive to citreoviridin. The mutation leading to aurovertin and citreoviridin resistance was rather selective, as the mutated ATPase was still inhibited by a number of other reagents: dicyclohexylcarbodiimide, sodium azide, *N*-ethoxycarbonyl 2-ethoxy-1,2-dihydroquinoline, and 4-chloro-7-nitrobenzofurazan (35).

TABLE 1. *Reconstitution of ATPase activity with BF_1 subunits from wild-type (AN180) and aurovertin-resistant (MA12) strains*[a]

Subunit combination	ATPase activity (μmol/min per mg of protein)		
	Control	+20 μM aurovertin	+5 mM sodium azide
$\alpha\beta^{MA12}\gamma\epsilon$	29	29.5	2.9
$\alpha^{MA12}\beta\gamma^{MA12}\epsilon$	41	13	1.0

[a] Subunits isolated as described by Vogel and Steinhart (42) were mixed in the proportion of 250 μg of α, γ, and ϵ to 250 μg of β and dialyzed for 12 h at 23°C against a reconstitution buffer (pH 6.1) made of 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 2 mM ATP, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. The mixture was chromatographed at 4°C on a Sepharose 6B column (1.5 by 90 cm) equilibrated with a solution of 50 mM Tris-hydrochloride, 2.5 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM ATP (pH 7.4), and 20% (vol/vol) methanol. The fractions were assayed for ATPase activity. Active fractions were pooled and used for the assays.

Linnett et al. (25) have reported that citreoviridin diacetate stimulates the ATPase activity of soluble F_1 from heart mitochondria. In accordance with this finding, we observed a slight, but reproducible, stimulation of *E. coli* BF_1 activity by acetyl aurovertin. It is noteworthy that the ATPase activity of the aurovertin-resistant MA12 mutant was also stimulated by aurovertin. At present we can offer no explanation for this dual effect, stimulatory and inhibitory, of aurovertin.

Interaction of aurovertin with either BF_1 or the isolated β subunit led to strong enhancement of aurovertin fluorescence, a feature probably related to a reduced mobility of bound aurovertin. This was also reported by Dunn and Futai (15). Aurovertin can therefore be used as a fluorescent probe of conformational changes in BF_1 (36) in the same manner as for F_1 (9, 10, 22). The changes in fluorescence intensity brought about by addition of ADP and ATP to the aurovertin-BF_1 complex were consistent with the existence of multiple nucleotide-binding sites on isolated BF_1. The first set of sites recognized by ADP (ATP) was responsible for the enhancement of fluorescence intensity. The apparent affinity for ADP (ATP) was close to that measured directly by using [^{14}C]ADP (6). The enhanced fluorescence intensity of the ADP-aurovertin-BF_1 complex was quenched by ATP, the ATP interaction being characterized by a K_d of 500 to 600 μM. This K_d value was in agreement with the $K_{m_{ATP}}$ for ATP hydrolysis; it most likely reflected the interaction of ATP with a specific conformation of the catalytic site of BF_1 adapted for ATP hydrolysis.

Upon freezing and thawing, BF_1 split reversibly to give a cluster of (α, γ, and ϵ) subunits and an isolated β subunit. The hybrid enzyme $\alpha\beta^{MA12}\gamma\epsilon$ obtained by in vitro complementation was not inhibited by aurovertin in contrast to the hybrid $\alpha^{MA12}\beta\gamma^{MA12}\epsilon^{MA12}$. This complementation assay was in accordance with the fluorescence data, which showed that subunit β in aurovertin-resistant BF_1 was unable to bind aurovertin.

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