Binding of [^{14}C]Aurovertin D to *Escherichia coli* F_1-ATPase and the Isolated β Subunit. Correlation with Inhibition of the ATPase Activity†

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ABSTRACT: [^{14}C]Aurovertin D with a specific radioactivity of 60×10^{12} dpm/mol was prepared by a multistep method involving the following sequence: protection of the two free -OH groups of aurovertin D by 5,6-dihydro-4-methoxy-2*H*-pyran, removal of the acetyl residue by saponification followed by replacement with a [^{14}C]acetyl group through reaction with [^{14}C]acetic anhydride, and finally elimination of the protecting residues. On the basis of the specific radioactivity of the ^{14}C-labeled aurovertin D and the incorporation of one [^{14}C]acetyl group per aurovertin D, an ϵ of 36 100 M^{-1} cm^{-1} for aurovertin D at 368 nm was calculated. The number of aurovertin binding sites on purified soluble ATPase solubilized from *Escherichia coli* (BF_1) was determined with [^{14}C]aurovertin D by equilibrium dialysis. When the medium was supplemented with ADP, ATP, or divalent cations, three binding sites per BF_1 could be demonstrated; one of them had a higher affinity for aurovertin D ($K_d \simeq 0.2$ μM) than the other two (K_d = 3–5 μM and 7–10 μM). In the absence of added ADP, ATP, or divalent cations, the two low-affinity sites and a fraction (only one-third) of the high-affinity site were titrated. The aurovertin D insensitive BF_1 isolated from an aurovertin D resistant mutant of *E. coli* (MA12) [Satre, M., Klein, G., & Vignais, P. V. (1978) *J. Bacteriol. 134*, 17–23] was found unable to bind [^{14}C]aurovertin D. The binding data obtained by the isotopic method were compared with those derived from the change of aurovertin fluorescence upon interaction with BF_1. By the latter method, only one high-affinity site for aurovertin D could be demonstrated. The isolated β subunit of BF_1 could bind [^{14}C]aurovertin D with a stoichiometry of 1 and a K_d of 5.8 μM; upon addition of ADP, the binding stoichiometry remained unchanged, but the affinity for aurovertin D was increased (K_d = 1.4 μM). A similar effect of ADP on the affinity of the isolated β subunit for aurovertin D was revealed by the fluorescence assay. Since aurovertin recognizes specifically the β subunit in BF_1 with a stoichiometry of 1 and each F_1 contains three aurovertin sites, it is concluded that there are three β subunits per BF_1. Occupancy of binding sites on BF_1 by aurovertin D was correlated with inhibition of ATPase activity. Aurovertin D was found to inhibit both BF_1 and BF_1–ATP. The affinity of aurovertin D for BF_1–ATP was higher than for BF_1 alone. The K_i value relative to the ternary complex, aurovertin–BF_1–ATP, 0.1–0.2 μM, was similar to the K_d value relative to the aurovertin high-affinity site of BF_1. These results indicate that binding of ATP to BF_1 facilitates the binding of aurovertin D to the enzyme and suggest that aurovertin D inhibits the ATPase activity of BF_1 primarily by binding to its high-affinity site.

Aurovertin D belongs to a family of natural products, the aurovertins, that are synthesized by the fungus *Calcarisporium arbuscula*. Aurovertin D was first isolated by Baldwin et al. (1964) and introduced by Lardy et al. (1964) as a tool to study the mechanism of oxidative phosphorylation in mammalian mitochondria where it was found to inhibit ATP synthesis linked to electron transport, $^{32}P_i$–ATP exchange, and ^{18}O exchange between P_i and ATP. Later, it was reported (Kagawa & Racker, 1966; Roberton et al., 1967) that aurovertin D inhibits soluble mitochondrial F_1[1] by formation of an aurovertin–F_1 complex (Lardy & Lin, 1969; Chang & Penefsky, 1973). Because this complex is fluorescent and its fluorescence is altered upon addition of nucleotides, P_i, and Mg^{2+}, aurovertin D was extensively used to monitor conformational changes of F_1 induced by these ligands [for a review, see Penefsky (1979)]. Aurovertin D also binds to *Escherichia coli* F_1 (BF_1) (Satre et al., 1978, 1980; Dunn & Futai, 1980; Bragg et al., 1982). In both mitochondrial and bacterial F_1, the liganded subunit is the β subunit (Douglas et al., 1977; Verschoor et al., 1977; Dunn & Futai, 1980; Satre et al., 1980). On the basis of the fluorescence enhancement of bound aurovertin D and its specific binding to the β subunit, a number of investigations have been directed to the study of the β subunit stoichiometry in F_1 [for a review, see Penefsky (1979)]. This approach was, however, hampered by difficulty in analysis of the fluorometric titration curves. Direct binding studies with radiolabeled aurovertin D were clearly needed; moreover, comparison of aurovertin titration data using fluorometry and radiolabeling would hopefully resolve ambiguities on the interpretation of fluorometric data.

In the present paper, we describe a preparation of ^{14}C-labeled aurovertin D and the binding parameters of [^{14}C]aurovertin D to BF_1. We took advantage of the existence of a mutant of *E. coli* whose F_1-ATPase was not inhibited by aurovertin D and did not respond to added aurovertin D by variations in fluorescence intensity (Satre et al., 1978, 1980). Binding studies carried out with purified BF_1 from the wild type and the mutant strain showed unambiguously the specific nature of the binding of [^{14}C]aurovertin D. The existence of three aurovertin binding sites of different affinities per BF_1 was demonstrated, and a correlation was established between the binding and inhibition properties of [^{14}C]aurovertin D.

Experimental Procedures

Materials

[^{14}C]Acetic anhydride labeled in the two carboxyl groups (54.8 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (Saclay, France), ATP, NADH, phosphoenol-

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[1] Abbreviations: F_1, soluble F_1-ATPase isolated from mitochondria; BF_1, soluble F_1-ATPase isolated from *E. coli*; BF_1–ATP, complex made by association of BF_1 with its substrate ATP; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate.

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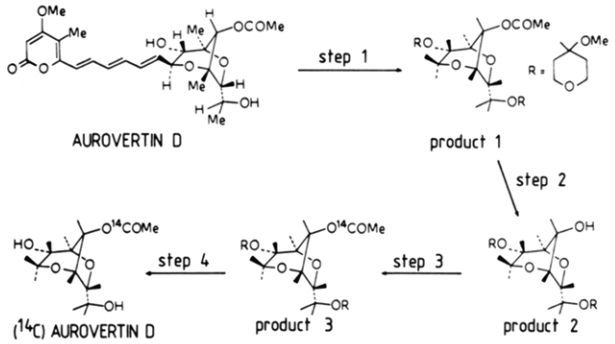


FIGURE 1: Scheme depicting steps involved in radiolabeling of aurovertin D. The two free-hydroxyl groups of aurovertin D were first protected with 5,6-dihydro-4-methoxy-2*H*-pyran (step 1). The acetyl group was then removed by saponification (step 2) and replaced by a [^{14}C]acetyl group (step 3). The two protecting groups were removed in step 4, leading to [^{14}C]aurovertin D.

pyruvate, pyruvate kinase, and lactate dehydrogenase were obtained from Boehringer, ADP was obtained from Sigma, and 5,6-dihydro-4-methoxy-2*H*-pyran was obtained from Aldrich. Pyridine (Merck) was distilled twice over KOH and ninhydrin and stored over CaH_2 at 4 °C before use.

Methods

Isolation of Aurovertin D. Aurovertin D was extracted and purified from the mycelium and growth medium of 3-week-old cultures of *Calcarisporium arbuscula* (NRRL 3705), essentially as described by Osselton et al. (1974). Aurovertin D was stored in methanolic solution protected from light at −20 °C, and its concentration was determined by using a molar absorption coefficient of 36 100 at 368 nm (see below).

Synthesis of [^{14}C]Aurovertin D. It has been briefly mentioned (Linnett & Beechey, 1979) that the acetyl group in aurovertin B could be replaced by other acyl esters by means of a protection–saponification–esterification–deprotection sequence in which the dihydropyran was used as a protecting reagent. On this basis, chemical radiolabeling of aurovertin D was performed by replacement of the unlabeled acetyl residue of aurovertin D by a [^{14}C]acetyl group from [^{14}C]acetic anhydride. The labeling procedure is summarized in Figure 1. All the reactions were carried out at room temperature. The first reaction (step 1) was directed at the protection of the free hydroxyl groups of aurovertin D. Instead of using dihydropyran as protecting agent as suggested by Linnett & Beechey (1979), we preferred 5,6-dihydro-4-methoxy-2*H*-pyran to avoid production of isomers (Reese et al., 1967). Purified aurovertin D (33 μmol) was dissolved in 0.55 mL of 5,6-dihydro-4-methoxy-2*H*-pyran with a crystal of *p*-toluenesulfonic acid as catalyst. After 1 h of reaction, product **1** obtained by condensation of the protecting group with aurovertin D (Figure 1) was purified by thin-layer chromatography on silica gel plate (60F-254, Merck) with a developping phase made of benzene and ethyl acetate (3/7 v/v). Product **1** was characterized by an R_f of 0.49 as compared to 0.31 for aurovertin D (Figure 2); it was extracted with acetone. Step 2 consisted of alkaline hydrolysis of the acetyl ester group of aurovertin D. Acetone was removed under partial vacuum in a rotary extractor, and the residue was dissolved in 2.7 mL of methanol to which 60 μL of 5 N NaOH was added. The reaction was allowed to proceed for 1 h, and then 8 mL of water was added. The products of the reaction were extracted with ethyl acetate and separated by thin-layer chromatography with the same phase as that used in step 1. The compound of R_f 0.21 (product **2**) was extracted with acetone. Acetone was removed under partial vacuum. The residue was solubilized in 200 μL of anhydrous pyridine to which [^{14}C]acetic anhydride (146 μmol, 54.8 Ci/mol) was added. The acetylation reaction (step 3) was allowed to proceed overnight. The mixture was then evaporated to dryness to remove the unreacted acetic anhydride, and 1 mL of water was added. The products were extracted with ethyl acetate and separated by thin-layer chromatography in the same phase as that used for step 1. The radiolabeled product **3**, characterized by an R_f of 0.49 identical with product **1**, was extracted from the plate with acetone (Figure 2). The final step (step 4) allowed the regeneration of aurovertin D by removal of the protecting residues. After removal of acetone under reduced pressure, product **3** was solubilized in 1 mL of methanol, and 15 μL of 12 N HCl was added. After 10 min of reaction, the mixture was chromatographed on a thin-layer plate in the same phase as that used in step 1. The compound migrating with an R_f of 0.31 corresponded to [^{14}C]aurovertin D; it was recovered by extraction with acetone. Acetone was removed, and the [^{14}C]aurovertin D was finally solubilized in methanol. The final yield of [^{14}C]aurovertin D ranged between 10 and 15%, with a specific radioactivity of 60×10^{12} dpm/mol.

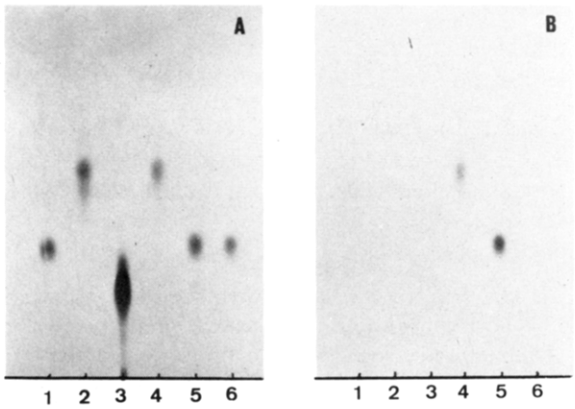


FIGURE 2: Thin-layer chromatography of [^{14}C]aurovertin D and intermediates of synthesis. The chromatography was carried out on a silica gel plate in benzene/ethyl acetate (3/7 v/v). The plate was UV photographed (A) and autoradiographed (B). Spots 1 and 6 (R_F 0.31) correspond to aurovertin D. Spots 2 (R_f 0.49), 3 (R_f 0.21), 4 (R_f 0.49), and 5 (R_f 0.31) correspond to products **1**, **2**, and **3** and [^{14}C]aurovertin D, respectively.

Characterization of [^{14}C]Aurovertin D. The purity of [^{14}C]aurovertin D was checked by high-performance liquid chromatography, using a μBondapak C_{18} column (Waters). Elution was carried out with a linear gradient of 0–100% aqueous methanol at a flow rate of 1 mL/min at 25 °C. Only one peak of radioactivity was found, corresponding to authentic aurovertin D. Radiolabeled aurovertin D showed the same UV spectrum as unlabeled aurovertin D and, in particular, the same absorbance ratio at 368 and 270 nm. Likewise, the fluorescence response to addition of BF_1 was identical, as well as the inhibitory efficiency with respect to the hydrolytic activity of BF_1 (same K_i).

Determination of Molar Extinction Coefficient (ε) of Aurovertin D. On the basis of the specific radioactivity of [^{14}C]acetic anhydride (120×10^{12} dpm/mol) and the incorporation of one [^{14}C]acetic group per molecule of aurovertin D, a molar absorption coefficient of 36 100 at 368 nm for aurovertin D was calculated.

BF_1 Purification. BF_1 was purified from *E. coli* K12 AN180 (Butlin et al., 1971) as described by Satre et al. (1979) except that the concentration step on Amicon XM 100 was replaced by precipitation with poly(ethylene glycol) and Mg^{2+} (Vogel & Steinhart, 1976). Purified BF_1 was stored at 0–4 °C in 50 mM Tris-HCl, 2.5 mM 2-mercaptoethanol, 2 mM

EDTA, 2 mM ATP, and 20% (v/v) methanol, pH 7.4, at a protein concentration of about 5–10 mg/mL. This ATPase preparation almost completely lacked the δ subunit. BF_1 from the aurovertin D resistant strain MA12 (Satre et al., 1978) was obtained by the same procedure. A molecular weight of 362 000 for BF_1 was used, on the basis of DNA sequencing data (Saraste et al., 1981) and of a subunit stoichiometry of $\alpha_3\beta_3\gamma\epsilon$ (omitting δ) (Bragg & Hou, 1975; Satre et al., 1982).

Purification of β Subunit from BF_1. BF_1 was dissociated according to the procedure of Dunn & Futai (1980). The dissociated BF_1 was chromatographed on a DEAE-cellulose column to yield the β subunit and an $\alpha\gamma\epsilon$ complex, as described by Vogel & Steinhart (1976). The detailed procedure was reported by Satre et al. (1982). The purity of the β fraction was checked by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Protein. Protein concentration was assayed by the Coomassie G250 Blue method (Bradford, 1976), bovine serum albumin being used as a standard. The Folin–Ciocalteu method (Zak & Cohen, 1961) gave identical results.

Fluorescence Assays. Fluorescence was measured in 2 mL of 0.25 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA, pH 7.4, in a Perkin-Elmer MPF 2A spectrofluorometer at 20 °C. The excitation wavelength was set at 365 nm and the emission wavelength at 470 nm. Prior to fluorescence measurements, the samples of BF_1 were filtrated through short columns of Sephadex G-50 (fine) equilibrated with the same medium as that used for fluorescence assay as described by Penefsky (1977).

[^{14}C]Aurovertin D Binding Assay. Binding of [^{14}C]aurovertin D to BF_1 was measured by equilibrium dialysis at 20 °C in a Dianorm apparatus equipped with microcells (2 × 250 μL) and Sartorius cellulose–acetate membranes (M_r cutoff 160 000) in a medium containing 0.25 M sucrose, 0.5 mM EDTA, and 10 mM Tris-HCl, final pH 7.4 (buffer I).

One half-cell was filled with 180 μL of the enzyme preparation obtained by centrifugation–filtration through a short Sephadex G-50 column equilibrated with buffer I; the other half-cell was filled with 180 μL of the dialysis buffer containing [^{14}C]aurovertin D; the same operation was conducted with increasing concentrations of [^{14}C]aurovertin D. Dialysis was carried out for 90 min under continuous rotation (12 rpm). Preliminary assays showed that dialysis equilibration with aurovertin D occurred in less than 60 min and that less than 5% of the BF_1 activity was lost in 4 h of dialysis; furthermore, it was verified that no protein escaped through the dialysis membrane. Radioactivity in the half-cell without protein corresponded to the free [^{14}C]aurovertin D, and in the other half-cell, it corresponded to bound plus free [^{14}C]aurovertin D. The radioactive samples (30–100 μL) were mixed with 1 mL of a mixture of water and methanol (1/1 v/v) and counted with 10 mL of a scintillation fluid (Patterson & Greene, 1965); the yield of counting was determined after addition of a [^{14}C]toluene internal standard obtained from the Laboratoire de Métrologie, Saclay, France.

Calculation of Binding Constants for Aurovertin Binding from Radioactivity Measurements. The curvilinear Scatchard plot (Scatchard, 1949) of the [^{14}C]aurovertin binding data was analyzed in terms of independent sites on F_1 with different affinities for aurovertin D. The number of sites and the K_d values of these sites were calculated after decomposition of the Scatchard plot into straight lines by the graphical method of Rosenthal (1967).

ATPase Assay. The ATPase activity of BF_1 was determined by spectrophotometry in the presence of a regenerating system for ATP at 28 °C. The reaction was started by addition of BF_1 to 2 mL of a medium consisting of 2 mM phosphoenolpyruvate, 25 μg/mL pyruvate kinase, 12 μg/mL lactate dehydrogenase, 0.2 mM NADH, 12 mM KCl, 10 mM Tris-HCl, pH 8.0, and ATP plus $MgCl_2$ at concentrations indicated in the legends of the figures. Oxidation of NADH linked to hydrolysis of ATP was monitored by recording the decrease in absorbancy at 340 nm. The rates were calculated from the period at which the kinetics became linear (i.e., 3–5 min after addition of BF_1). ATPase activities were expressed in units per milligram (units/mg), a unit being equal to 1 μmol of ATP hydrolyzed/min. Calculations of the Mg–ATP complex concentrations at a given fixed free Mg^{2+} concentration were performed as described by Morrison (1979).

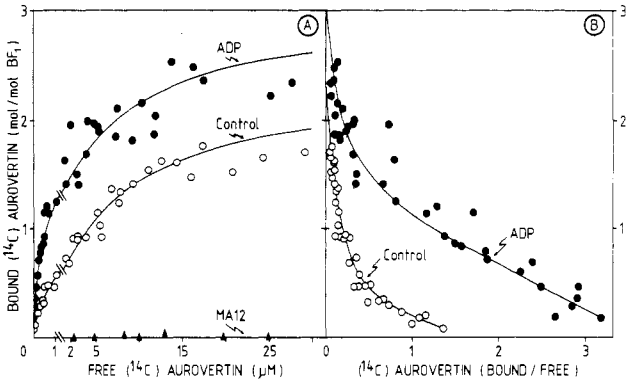


FIGURE 3: Binding of [^{14}C]aurovertin D to BF_1 in the presence or absence of ADP. The binding of [^{14}C]aurovertin D was measured by equilibrium dialysis, as described under Methods, in a medium consisting of 0.25 M sucrose, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4. A number of assays were carried out at 20 °C, using a constant concentration of BF_1, 0.7 μM, and increasing concentrations of [^{14}C]aurovertin D. No added ADP (○); 500 μM ADP (●). (A) Direct binding curve; (B) Scatchard plot. (▲) Direct binding curve for BF_1 (0.7 μM) isolated from the aurovertin-resistant mutant MA12.

Results

Binding of [^{14}C]Aurovertin D to BF_1. Binding assays with [^{14}C]aurovertin D were carried out with the aurovertin-sensitive BF_1 isolated from the wild strain of *E. coli* K12 AN180 and from the aurovertin-insensitive BF_1 isolated from the mutant MA12. In preliminary experiments, it was checked that up to 2 μM the concentration of BF_1 could be varied without any significant change in the binding affinity and capacity for aurovertin D. Incubation of [^{14}C]aurovertin D with the aurovertin-sensitive BF_1 in a medium supplemented with 0.5 mM ADP resulted in the maximal binding of 3 mol of [^{14}C]aurovertin D/mol of BF_1, indicating three aurovertin binding sites per F_1 (Figure 3A); the corresponding Scatchard plot was curvilinear (Figure 3B). Two hypotheses at least may be formulated to explain this curvilinearity: (1) the three sites are independent, and (2) the three sites are interacting with negative cooperativity. On the basis of the hypothesis that curvilinearity reflects distinct binding sites, the Scatchard plot was decomposed by the graphical method of Rosenthal (1967) to yield three independent classes of sites: a class of high-affinity sites with a K_d value of about 0.2 μM and two classes of lower affinity sites with K_d values of 3–5 μM and 7–10 μM, respectively. Similar binding curves were obtained when the titrations were performed in the presence of 4 mM ATP or 3 mM $MgCl_2$. The binding parameters under these different conditions are listed in Table I. When the binding of aurovertin D to BF_1 was performed in a medium devoid of nucleotides or Mg^{2+}, the amount of high-affinity sites ($K_d \simeq 0.2$ μM) decreased from 1 to 0.3; the two sites of lower affinity

Table I: Binding Parameters of [^{14}C]Aurovertin D to BF_1

addition	no.[a] of high-affinity sites and K_d values (μM)[b]	no.[a] of low-affinity sites and K_d values (μM)[b]
ADP[c]	1 (0.19–0.21)	2 (3–5 and 7–10)
ATP[c]	1 (0.21–0.24)	2 (3–5 and 7–10)
$MgCl_2$[c]	1 (0.21–0.24)	2 (3–5 and 7–10)
none	0.3 (0.16–0.20)	2 (3–5 and 7–10)

[a] The integral numbers of sites of high affinity (except in the absence of $MgCl_2$ and nucleotide) and low affinity were used as a basis for the mathematical analysis of the [^{14}C]aurovertin binding curve. Titrations were done at least in triplicate. [b] The K_d values are in parentheses. [c] The final concentrations used were 0.5 mM ADP, 4 mM ATP, and 3 mM $MgCl_2$.

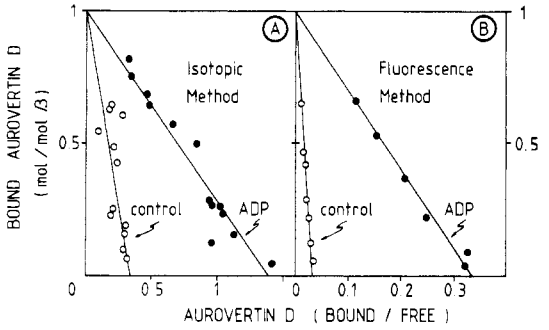


FIGURE 4: (A) Binding of [^{14}C]aurovertin D to the isolated β subunit in the presence or absence of ADP. The binding of [^{14}C]aurovertin to β (final concentration 2 μM) was measured by equilibrium dialysis in the same medium as in Figure 3: without ADP (○); with 500 μM ADP (●). (B) Binding of aurovertin D to the isolated β subunit (final concentration 0.2 μM) as assessed by fluorescence measurement: without ADP (○); with 500 μM ADP (●). Note the different scales of abscissa for panels A and B.

(K_d = 3–5 μM and 7–10 μM) were unaltered. Extrapolation of the Scatchard plot, in the absence of nucleotides or Mg^{2+}, was, however, not accurate enough to determine whether the three binding sites were still occupied, i.e., whether the partial loss of aurovertin high-affinity binding sites was compensated by an increase of the amount of low-affinity sites.

With the same medium as that used for the binding of [^{14}C]aurovertin to sensitive BF_1, there was no significant binding of [^{14}C]aurovertin D to BF_1 prepared from the MA12 aurovertin-resistant mutant (see Methods). This held for the different conditions used, i.e., in the absence or presence of ADP, ATP, or Mg^{2+}.

Binding of [^{14}C]Aurovertin D to Isolated β Subunits. The binding parameters of [^{14}C]aurovertin D relative to the isolated β subunit were studied by the same equilibrium dialysis technique as that described for the binding of [^{14}C]aurovertin D to BF_1; a cellulose membrane Spectrapor 2 with an M_r cutoff of 12 000–14 000 was used. One aurovertin binding site per isolated β subunit was found; the K_d was 5.8 μM in the absence of ADP and 1.4 μM in the presence of 0.5 mM ADP (Figure 4A).

Aurovertin D Binding Assessed by Changes of Fluorescence Intensity. Binding of aurovertin D to the mitochondrial F_1 has been quantitatively assessed on the basis of the fluorescence increase of aurovertin D upon binding to F_1 (Chang & Penefsky, 1973; Van de Stadt et al., 1974; Müller et al., 1977). It was therefore desirable to apply the fluorescence technique to the measurement of aurovertin binding to BF_1 and to compare the data with those obtained by the radioactivity technique. To measure the binding parameters of aurovertin D to BF_1 and the isolated β subunit by fluorometry, we followed the same rationale as that adopted by Müller et al.

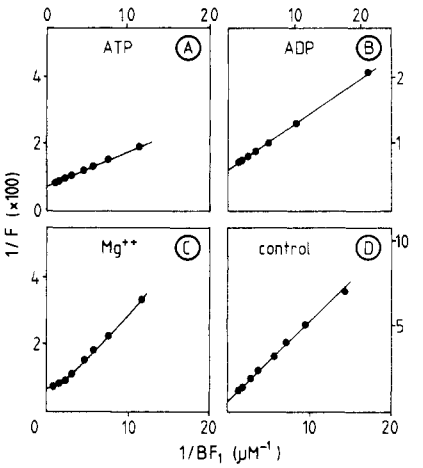


FIGURE 5: Determination of fluorescence coefficient of bound aurovertin D. The reciprocal of the fluorescence intensity of bound aurovertin at a fixed concentration of aurovertin was plotted as a function of the reciprocal of BF_1 concentration (cf. Results). The medium used is described under Methods. ATP (curve A), ADP (curve B), and $MgCl_2$ (curve C) were added at the final concentrations of 4, 0.5, and 3 mM, respectively. Curve D corresponds to a control assay in the absence of added nucleotides or Mg ions. The aurovertin concentration was 65 × 10^{-3} μM in curves A, B, and D and 163 × 10^{-3} μM in curve C. The fluorescence coefficients, α_b, were calculated as described under Results; their values are as follows: 2100 with ATP, 2500 with ADP, 890 with $MgCl_2$, 2600 in the absence of added ligand.

(1977) for the mitochondrial F_1. The aurovertin D concentration bound to the whole enzyme or the isolated β subunit was expressed by

$$a_b = (F_{obsd} - \alpha_f a)/(\alpha_b - \alpha_f) \quad (1)$$

where F_{obsd} is the fluorescence intensity, resulting from the mixing of aurovertin D and BF_1, α_f and α_b are fluorescence coefficients [arbitrary units (μM^{-1})] for free and bound aurovertin D, and a_b and a are the concentrations of bound and total aurovertin D (μM). α_f being less than 3% of α_b can be omitted, and eq 1 is therefore reduced to

$$a_b = (F_{obsd} - \alpha_f a)/\alpha_b = F_{cor}/\alpha_b \quad (2)$$

where $\alpha_f a$ is the measured fluorescence in the absence of BF_1 and α_b is derived by extrapolating to infinite concentration of protein the reciprocal of the fluorescence intensity vs. the reciprocal of the protein concentration for a given concentration of aurovertin D (Figure 5). This method introduced a large uncertainty when the reciprocal plot was curvilinear as illustrated for the medium supplemented in $MgCl_2$. The titration was performed at a given concentration of BF_1 and increasing concentrations of aurovertin D; the Scatchard representations of binding data in the presence of ADP, ATP, or $MgCl_2$ and in the absence of ligands (control) are given in Figure 6. In the presence of either ADP, ATP, or Mg^{2+}, one aurovertin binding site per BF_1 was titrated with a K_d value of 0.09–0.18 μM, which corresponded to the high-affinity site found by the radioactivity method. In the absence of added ligands (control), aurovertin binding was markedly decreased; in this case the curvilinearity of the Scatchard plot pointed to the existence of about 0.3 site of K_d = 0.24 μM and possibly another (other) site(s) of lower affinity (K_d > 1 μM). It may be recalled that upon addition of aurovertin D to BF_1 isolated from the aurovertin mutant MA12, no fluorescence enhancement is detected (Satre et al., 1978, 1980)

Fluorescence titrations of the aurovertin binding sites on the isolated β subunit were carried out as for the native BF_1. In

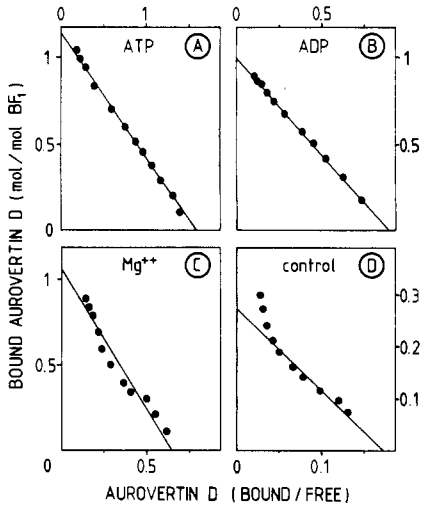


FIGURE 6: Scatchard plot of binding data of aurovertin D to BF_1 as assessed by fluorescence measurement. The medium used is described under Methods. ATP (curve A), ADP (curve B), and $MgCl_2$ (curve C) were used at the final concentrations of 4, 0.5, and 3 mM, respectively. Curve D corresponds to the control. The final concentrations of BF_1 were in the range of 0.12–0.18 μM. The fluorescence coefficients of bound aurovertin determined in Figure 5 were used to calculate the binding data of Figure 6. Note the different scales of abscissa for curves A–D.

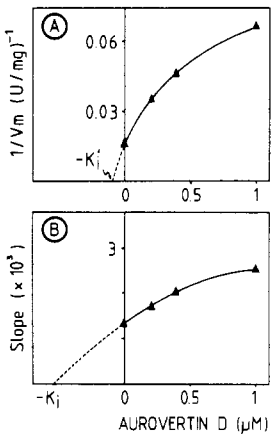


FIGURE 7: Inhibitory effect of aurovertin D on BF_1 at low Mg^{2+} concentration. The conditions of ATP hydrolysis by BF_1 are given under Methods. The free-Mg^{2+} concentration was 10 μM. (A) Determination of the dissociation constant of the complex aurovertin–BF_1–substrate (K_i'). The $1/V_{max}$ values that were obtained from the reciprocal plots of v vs. [Mg–ATP] for different aurovertin concentrations were plotted against aurovertin concentrations. (B) Determination of the dissociation constant of the complex aurovertin–BF_1 (K_i). The slope values that were measured from the reciprocal plots of v vs. [Mg–ATP] for different aurovertin concentrations were plotted against aurovertin concentrations.

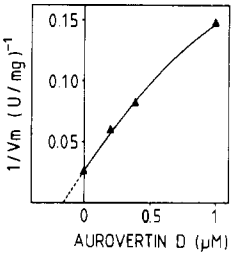


FIGURE 8: Inhibitory effect of aurovertin D on BF_1 at high Mg^{2+} concentration. Determination of the dissociation constant of the complex aurovertin–BF_1. Same conditions as in Figure 7 except that the free-Mg^{2+} concentration was maintained at 500 μM. The $1/V_{max}$ values obtained from the reciprocal plots of v vs. [Mg–ATP] for different aurovertin concentrations were plotted against aurovertin concentrations.

the absence of nucleotides, the maximal fluorescence of aurovertin D bound to the β subunit was 2–3 times lower than that of aurovertin D bound to BF_1. One aurovertin binding site per β subunit was found; its K_d was about 2.5 μM (Figure 4B), a value very close to that, 3.1 μM, reported by Dunn & Futai (1980). Affinity of the isolated β subunit for aurovertin was enhanced upon addition of ADP; for 0.5 mM ADP, the K_d vas 0.6 μM (Figure 4B). The half-maximal effect of ADP was obtained at 10–20 μM ADP.

Relation between Aurovertin Binding and Inhibition of BF_1 ATPase Activity. As shown for mitochondrial F_1 (Lardy, 1961; Roberton et al., 1967), inhibition by aurovertin D is never complete, even at exceedingly high concentrations of aurovertin. By extrapolation of the percentage of inhibition of BF_1 ATPase activity at infinite concentration of aurovertin, it was found that BF_1 retained about 15% of its activity, in agreement with the data reported by Satre et al., (1978). Although one could not rule out the possibility that the population of BF_1 molecules is heterogeneous and that a fraction of it is insensitive to aurovertin, a more plausible explanation was that the aurovertin–BF_1 complex is not totally inactive. In our experimental conditions, BF_1 behaved as a Michaelian enzyme, without apparent cooperative interaction. Contrary to the mitochondrial F_1, the rate of ATP hydrolysis by BF_1 was not influenced by the presence of the anions SO_4^{2-} and HCO_3^- (not shown).

When free Mg^{2+} was maintained at low concentration (10 μM), the apparent K_M of BF_1 for the Mg–ATP complex and the V_{max} were decreased by aurovertin D to different extents. The reciprocal plots of the rate vs. the Mg–ATP concentration for increasing concentrations of aurovertin intersected below the base line. Further, the secondary plot of $1/V_{max}$ for different fixed concentrations of Mg–ATP vs. aurovertin concentrations showed a downward curvilinearity (Figure 7A). These data are typical of a partial mixed inhibition in which the aurovertin–BF_1–ATP complex breaks down more slowly than does the BF_1–ATP complex to generate ADP and P_i. The intercept of the curve on the base line gave the K_i' value for the ternary complex BF_1–ATP–aurovertin, namely, 0.1 μM. The plot of the slopes of the Lineweaver–Burk graph vs. aurovertin concentration was also curved (Figure 7B); by extrapolation on the base line, a value of 0.5 μM for the K_i relative to the binary complex aurovertin–BF_1 obtained.

When free Mg^{2+} was maintained at high concentration (500 μM), i.e., when nearly all ATP was in the form of the Mg–ATP complex, aurovertin D behaved as a typical noncompetitive inhibitor. In this case, K_i and K_i' were virtually equal. The secondary plot of $1/V_{max}$ vs. aurovertin concentration was however curvilinear (Figure 8), indicating that, at difference with a pure noncompetitive inhibition, the aurovertin–BF_1–ATP complex can break down at low rate to generate the products ADP and P_i. Extrapolation of the plot to the base line yielded a K_i value of 0.15 μM for aurovertin.

Discussion

Aurovertin B was biosynthetically radiolabeled (Linnett et al., 1977; Linnett & Beechey, 1979) by growing *C. arbuscula* in a medium supplemented with [*methyl*-^{3}H]methionine. However, no detailed binding studies were presented. A possible difficulty inherent to the biosynthetic labeling is the low specific radioactivity that can be achieved as compared to the chemical radiolabeling. To our knowledge, the present work represents the first preparation of [^{14}C]aurovertin D obtained by chemical radiolabeling that allows the obtention

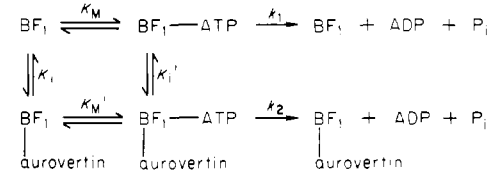
of a high specific radioactivity compatible with binding studies with isolated F_1-ATPases.

This paper presents a comparative study concerning two methods of determination of the binding parameters of aurovertin to BF_1 and the isolated β subunits. One is the direct isotopic measurement based on the use of [^{14}C]aurovertin D; in the other, binding of aurovertin was assessed by fluorometry, on the basis of the enhancement of the aurovertin fluorescence upon binding to BF_1 (Satre et al., 1980; Dunn & Futai, 1980). With the whole molecule of BF_1, three sites, one of high affinity and two of low affinity, were titrated with [^{14}C]aurovertin D; only one binding site could be demonstrated by the fluorescence technique. The use of [^{14}C]aurovertin D allows, therefore, the detection of low-affinity sites that escaped fluorescence measurements. On the other hand, aurovertin D was found to bind to the isolated β subunit by both the isotopic and fluorometric techniques. However, as mentioned under Results, the interpretation of the fluorometric data was considerably hampered by the high degree of uncertainty in the extrapolation of the fluorescence intensity at infinite concentration of protein; for this reason, the isotopic technique was more reliable.

Before discussing the aurovertin binding data, it might be interesting to mention some inconveniences inherent to the fluorometric technique. (1) As stated above, measurement of binding sites requires the determination of the fluorescence coefficient at infinite protein concentration; the curvilinearity of some plots makes the extrapolation inaccurate. (2) Some energy transfer may occur between bound molecules of aurovertin D, resulting in underestimation of the number of binding sites. (3) The fluorescent coefficient at infinite protein concentration appears to reflect only the interaction of aurovertin D with the site of highest affinity. (4) It may be added that a difficulty in the determination of the number of aurovertin binding sites comes from ambiguous determination of the concentration of aurovertin D that is routinely calculated from the molar absorption coefficient ϵ. The ϵ values reported in literature for aurovertin D are 42 700 at 367.5 nm (Baldwin et al., 1964), 28 500 at 367.5 nm (Müller et al., 1977), 38 500 at 369 nm (Linnett & Beechey, 1979), 35 100 at 368 nm (Satre et al., 1980). Mulheirn et al. (1974) used a value of 34 400 at 372 nm for a closely related molecule, aurovertin B. The two extreme ϵ values 42 700 and 28 500 differ by a factor of 1.5; consequently, the calculated number of aurovertin binding sites may vary by a factor of 1.5, depending on the ϵ value used to calculate the aurovertin concentration. With [^{14}C]aurovertin D, it was possible to circumvent this difficulty. Starting from [^{14}C]acetic anhydride equally labeled in the two carboxyl groups, the concentration of [^{14}C]aurovertin D was accurately determined, since the specific radioactivity of [^{14}C]aurovertin D is just half that of [^{14}C]acetic anhydride. The derived molar ϵ for [^{14}C]aurovertin D was 36 100 at 368 nm.

A few aspects of the isotopic method to investigate the binding parameters of aurovertin D deserve consideration. (1) The structure of [^{14}C]aurovertin D is identical with that of unlabeled aurovertin D since the radiolabeling consists in the replacement of an unlabeled acetyl group by a radiolabeled one. (2) Equilibrium dialysis allows investigation of both high- and low-affinity sites. One might argue that the long period of incubation required for equilibration of bound and free [^{14}C]aurovertin D in the two compartments of the dialysis cell might be deleterious to BF_1. In the period of 90 min required for full equilibration, less than 5% of the enzyme activity was lost, ruling out any dissociation of BF_1. Unspecific binding of [^{14}C]aurovertin D was also ruled out by the absence of detectable binding of [^{14}C]aurovertin D to BF_1 isolated from the aurovertin-resistant mutant MA12. As previously shown (Satre et al., 1978, 1980), the ATPase of BF_1 from the MA12 mutant is resistant to aurovertin D, and there is no change of fluorescence upon addition of aurovertin D to BF_1 from MA12.

Scheme I



One may wonder why the fluorescence technique allows only the detection of the aurovertin high-affinity site and not that of the low-affinity sites. As discussed by Chang & Penefsky (1973), aurovertin fluorescence depends on the rigidity imposed upon the fluorophore in the F_1 specific site, and this rigidity in turn clearly depends on the affinity binding so that only the aurovertin molecules bound with high affinity will fluoresce with high intensity.

An interesting aspect of the binding studies with [^{14}C]aurovertin D pertains to the stoichiometry of the β subunit in BF_1. On the basis of the fact that there are three binding sites for [^{14}C]aurovertin D per BF_1 and that [^{14}C]aurovertin D binds exclusively to the β subunit of BF_1 (Dunn & Futai, 1980; Satre et al., 1980) one is led to conclude that there are three β subunits per BF_1. This is in accordance with the β_3 stoichiometry as proposed by Bragg & Hou (1975), Dunn & Futai (1980), and Satre et al. (1982).

The binding of aurovertin D to BF_1 was compared to its inhibitory effect on the ATPase activity of BF_1. In the case of the mitochondrial F_1, an uncompetitive type of inhibition of the hydrolytic activity of F_1 was reported when the substrates were Mg–ATP (Chang & Penefsky, 1973) and Mg–ITP (Ebel & Lardy, 1975). However, a complicating factor in the evaluation of the inhibitory data with Mg–ATP as substrate appeared to be the stimulatory effect of bicarbonate on the ATPase activity of F_1 (Ebel & Lardy, 1975). It may be recalled that BF_1 is not significantly activated by bicarbonate, which makes the evaluation of kinetic data easier. Two typical cases related to $MgCl_2$ concentration were considered. At low free-$MgCl_2$ concentration (<10 μM), the inhibition of the ATPase activity of BF_1 by aurovertin D was of a mixed type; it was partially noncompetitive at high free-$MgCl_2$ concentration (>500 μM). Since BF_1 behaves as a Michaelian enzyme, the kinetic data could be fitted by a simple model based on a single substrate–enzyme complex inhibited by the binding of a single molecule of inhibitor. The inhibition data were compatible with the binding of aurovertin either to the free enzyme to form the binary complex aurovertin–BF_1 or to BF_1–ATP to form the ternary complex aurovertin–BF_1–ATP. The latter complex may be able, like BF_1–ATP, to generate ADP and P_i by hydrolysis, but with a much lower rate constant. This is illustrated in Scheme I, where the kinetic constant k_2 is much lower than k_1. As mentioned under Results, the affinity of BF_1 for ATP is increased by aurovertin binding ($K_M' < K_M$). A decrease of K_M for ATP in the presence of aurovertin D was observed by Mitchell & Moyle (1970) in the case of the membrane-bound mitochondrial F_1. Similarly, the K_d for the binding of ATP to BF_1 was found to be lower in the presence of aurovertin D (Wise et al., 1981). In the present paper, at low concentrations of free Mg^{2+} (10 μM), the K_i' value for aurovertin D in the

ternary complex aurovertin–BF_1–ATP was found to be 0.10 μM, which is close to the K_d value of the aurovertin high-affinity site in the presence of ATP, ADP, or $MgCl_2$. On the other hand, the K_i value relative to the binding of aurovertin D to free BF_1 was 0.5 μM. These kinetic data are in good agreement with binding data showing that the aurovertin high-affinity site requires ATP, ADP, or $MgCl_2$ to be fully expressed.

When the free-Mg^{2+} concentration was raised to 500 μM, the inhibition of the ATPase activity of BF_1 became typically noncompetitive, with, however, a nonnegligible rate of breakdown of the ternary complex aurovertin–BF_1–ATP. In this case and in contrast with the preceding one, K_i' was equal to K_i with values close to 0.10 μM. This may indicate that free BF_1 with an excess of Mg^{2+} undergoes a conformational change that increases its affinity for aurovertin D. Consistent with this idea is the finding that, in the presence of Mg^{2+} alone, without ADP or ATP, an aurovertin high-affinity site is revealed.

At least two possible mechanisms can be put forward to explain the different binding affinities of each of the three β subunits of BF_1 with respect to aurovertin D. (1) The three aurovertin sites are independent and heterogeneous; this difference in affinity for aurovertin exists prior to addition of aurovertin. Should aurovertin site heterogeneity exist, it would be most likely associated with a structural heterogeneity of the β subunits, which is not the case; a decisive criterion for homogeneous β subunits is that the β subunit is coded by one gene only in the *unc* operon (Hansen et al., 1981; Kanazawa et al., 1981; Saraste et al., 1981). (2) The three aurovertin sites are homogeneous in the absence of added ligand. Binding of aurovertin D to one of the three β subunits induces a change of conformation in the whole set of β subunits so that the affinity for aurovertin of the first liganded β subunits is higher than that of the other two. This site–site interaction that results in negative cooperativity may be an intrinsic property of the enzyme; in fact, in the case of the mitochondrial F_1, there is a number of kinetic data that can be explained by the fact that the three apparently identical β subunits of F_1, considered as catalytic subunits, are nonequivalent in the catalytic cycle (Cross et al., 1982; Gresser et al., 1982). Another explanation for the nonequivalence of aurovertin sites in identical β subunits integrated in the F_1 molecule is that each of the β subunits is characterized by a specific contact with the minor subunits of the enzyme, i.e., γ and ϵ in the case of the present preparation of BF_1. The specific contacts would provide a means by which the three β subunits assume different specific conformations responsible for the difference in aurovertin affinities. Specific contacts between each of the β subunits and the minor subunits γ, δ, and ϵ in the case of the mitochondrial F_1 are in fact strongly suggested by recent X-ray crystallographic studies (Amzel et al., 1982).

Registry No. ATPase, 9000-83-3; ADP, 58-64-0; aurovertin D, 65256-31-7; [^{14}C]aurovertin D, 85719-54-6.

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Aurovertin Binding Sites on Beef Heart Mitochondrial F_1-ATPase. Study with [^{14}C]Aurovertin D of the Binding Stoichiometry and of the Interaction between Aurovertin and the Natural ATPase Inhibitor for Binding to F_1†

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ABSTRACT: Chemically ^{14}C-labeled aurovertin D [Issartel, J. P., Klein, G., Satre, M., & Vignais, P. V. (1983) *Biochemistry* (preceding paper in this issue)] was used to study directly the interaction of aurovertin D with isolated beef heart mitochondrial F_1-ATPase by an equilibrium dialysis technique. With this assay, it was shown that each F_1 possesses three aurovertin sites; one of them had a much higher affinity than the other two (K_d = 0.2–0.3 μM vs. 3–5 μM). Isolated β subunit was found to bind [^{14}C]aurovertin D with a K_d of about 1 μM and a stoichiometry of 1 mol of aurovertin/mol of β subunit. As each F_1 binds three molecules of [^{14}C]aurovertin D, it follows that F_1 contains three β subunits. The aurovertin binding capacity of F_1 was not modified by addition of ADP or ATP. On the other hand, the binding affinity of F_1 for [^{14}C]aurovertin D was more affected by ATP than by ADP. In the presence of ATP, two relatively high-affinity sites ($K_d \simeq 0.7$ μM) and one low-affinity site ($K_d \simeq 5$ μM) were titrated. The binding data obtained by the isotopic technique were compared with those calculated from the classical fluorescence assay. The latter assay allowed the detection of only one high-affinity site ($K_d \simeq 0.2$ μM) and one low-affinity site ($K_d \simeq 5$ μM); upon addition of ATP, two sites of high affinity ($K_d \simeq 0.3$ μM) but none of low affinity were detected. The effect of the natural ATPase inhibitor, IF_1, on the binding of [^{14}C]aurovertin D was followed under conditions that allowed nearly full inhibition of the ATPase activity of F_1 by IF_1; this corresponds to the binding of 1 molecule of IF_1 to 1 molecule of F_1 at the level of the β subunit [Klein, G., Satre, M., Dianoux, A.-C., & Vignais, P. V. (1980) *Biochemistry 19*, 2919–2925]. Under these conditions the binding affinity of all three sites of F_1 for [^{14}C]aurovertin D was strongly decreased.

In the preceding paper (Issartel et al., 1983), a procedure for the chemical radiolabeling of aurovertin D was described; appropriate binding assays with *Escherichia coli* BF_1[1] and an aurovertin-insensitive mutant were reported, demonstrating the specific character of [^{14}C]aurovertin D binding to BF_1. The aim of the present work was to apply the same isotopic assay to the study of the interaction of aurovertin D with mitochondrial F_1. So far, all attempts to analyze the binding features of aurovertin D to mitochondrial F_1 have been based on the enhancement of the fluorescence of the aurovertin molecule upon binding to F_1 (Lardy & Lin, 1969). The number of moles of bound aurovertin per mole of F_1 was found to be 1 or 2 depending on experimental conditions (Lardy & Lin, 1969; Lardy & Lambeth, 1972; Bertina et al., 1973; Chang & Penefsky, 1973; Müller et al., 1977; Berden & Verschoor, 1978; Stutterheim et al., 1980). It was later demonstrated on the basis of the fluorescence test that aurovertin D binds in a 1 to 1 stoichiometry to the β subunit in beef heart F_1 (Verschoor et al., 1977) and yeast F_1 (Douglas et al., 1977; Stutterheim et al., 1980). As there was one binding site per β subunit and a maximal number of 2 mol of aurovertin bound per mol of F_1 from beef heart or yeast, Verschoor et al. (1977) and Stutterheim et al. (1980) concluded that each F_1 contained two β subunits.

The direct titration of the aurovertin site of F_1 with [^{14}C]aurovertin D described here shows unambiguously that the beef heart isolated β subunit binds 1 aurovertin molecule and that each beef heart F_1 contains three aurovertin binding sites of different affinities, leading to the conclusion that beef heart F_1 contains three β subunits. As an illustration of the use of [^{14}C]aurovertin D, we present a study of the effect of the natural ATPase inhibitor (Pullman & Monroy, 1963) on aurovertin binding to beef heart F_1.

Experimental Procedures

Biological Preparations. Beef heart mitochondria were isolated by the method of Smith (1967). Beef heart mitochondrial F_1 was prepared and stored as an ammonium sulfate precipitate as described by Knowles & Penefsky (1972). Before use, the suspension was centrifuged; the pellet was solubilized in the equilibrium dialysis buffer (see below) and desalted by the centrifugation–filtration method of Penefsky (1977). Molar concentrations of purified F_1 were calculated on the basis of an M_r of 360 000 (Lambeth et al., 1971). The natural ATPase inhibitor (IF_1) was prepared by the method of Horstman & Racker (1970).

Preparation of Isolated β Subunit. The procedure of Dunn & Futai (1980) described for the dissociation of *E. coli* ATPase was applied to the mitochondrial F_1-ATPase. All op-

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[1] Abbreviations: F_1, beef heart soluble mitochondrial ATPase; BF_1, soluble F_1-ATPase isolated from *E. coli*; IF_1, beef heart ATPase protein inhibitor; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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