Aurovertin, a Fluorescent Probe of Conformational Change in Beef Heart Mitochondrial Adenosine Triphosphatase*

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SUMMARY

Formation of a complex between aurovertin and soluble mitochondrial adenosine triphosphatase (F₁) was accompanied by a 55-fold enhancement of fluorescence, an increase in the polarization of fluorescence from 0.273 to 0.375 for the free form and a decrease in absorption at 366 nm. The fluorescence intensity of the complex was partially quenched by addition of ATP or Mg²⁺ and enhanced by ADP. Two binding sites for aurovertin were found on F₁ in the presence of ATP and one site in the presence of either ADP, Mg²⁺, or dilute buffer. The dissociation constants of the fluorescent complex were 0.013, 0.04 µM in the presence of ATP, ADP, Mg²⁺, or in buffer, respectively. It is proposed that of the two binding sites for aurovertin on F₁, only one site participates in inhibition of ATPase activity.

Addition of succinate to a fluorescent complex of aurovertin and submitochondrial particles gave rise to an enhancement of fluorescence which depended on maintenance of an energized state. The changes in fluorescence of bound aurovertin were interpreted in terms of changes in the conformational state of the ATPase.

The antibiotic aurovertin was first introduced as a tool for the study of oxidative phosphorylation by Lardy et al. (1) who established that this compound was a potent inhibitor of oxidative phosphorylation, the 32P-ATP exchange reaction, and the exchange of O₂ between the oxygen of P₃ and ATP catalyzed by rat liver mitochondria. In addition, aurovertin inhibited the hydrolysis of ATP stimulated by some uncouplers (1). Subsequently, it was found by Lenaz (2) and Lee and Ernster (3) that the forward reaction of ATP synthesis in oxidative phosphorylation was much more sensitive to aurovertin inhibition than the reverse energy transfer reactions from the respiratory chain to the terminal transphosphorylation step and suggested that aurovertin acted on only one pathway, at a point between the respiratory chain and the site of action of oligomycin. However, Lee and Ernster (3) and Robertson et al. (4) have proposed that aurovertin acted on the ATP side of the oligomycin-sensitive site.

The observation that aurovertin inhibited soluble mitochondrial ATPase (4) and formed stoichiometric fluorescent complexes with F₁ (5) prompted an examination of the interaction of this fluorophore with both soluble and membrane-bound mitochondrial ATPase. This paper provides evidence in support of the conclusion that aurovertin fluorescence is responsive to conformational changes in mitochondrial ATPase.

MATERIALS AND METHODS

Materials—ATP, Tris-ATP (metal-free), and DPNH were purchased from Sigma. ADP was obtained from Worthington. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer-Mannheim Corporation. Solvents and other chemicals were of the highest purity obtainable and were used without further purification. Aurovertin was a generous gift from Dr. Henry A. Lardy, University of Wisconsin.

Preparation of F₁—F₁ was prepared as described previously (6). The preparations used in this study exhibited a specific activity which varied between 100 and 120 units per mg. A molecular weight for F₁ of 347,000 (7) was used in binding calculations. Samples of the enzyme were freed of ammonium sulfate as follows. An aliquot of the ammonium sulfate suspension of the enzyme was centrifuged in the cold, and, after removal of the supernatant, the pellet was dissolved at room temperature by adding 0.5 ml of Buffer I (0.25 M sucrose, 2 mM EDTA, and 50 mM Tris sulfate, pH 8.0) containing 4 mM ATP. The sample of enzyme was clarified by low speed centrifugation if necessary and then applied to a column of Sephadex G-50 (1 x 23 cm) which had been equilibrated with the same buffer. The column was eluted with Buffer I-ATP, and the enzyme was collected in a volume of approximately 2 ml. The distribution of ammonium sulfate in the column eluate was monitored with the aid of Nessler's reagent.

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The abbreviations used are: F₁, soluble mitochondrial ATPase; ETPH, phosphorylating submitochondrial particles.

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unchanged by this procedure. F₁ was prepared in Buffer I containing ADP by the same procedure except that 1 mM ADP was substituted for ATP.

Samples of F₁, with a low content of adenine nucleotides were prepared as follows. About 10 ml of a suspension of Dowex 2-X₄ (hydroxide form) in Buffer I was brought to pH 8 by the addition of 1 N HCl. This material was used to prepare a column (1 X 3 cm) which was then washed with Buffer I. A sample of F₁, freed of ammonium sulfate in Buffer I containing ATP as described above, was applied to the column, and the enzyme was collected in a volume of about 3 ml. The ratio of the absorbance at 280 nm to that at 260 nm ranged between 1.33 and 1.47. The enzyme retained 80 to 90% of the original specific activity and was stable for about 5 hours at room temperature. However, on further standing, the solution lost activity and became turbid, and at higher concentrations (5 to 10 mg per ml) precipitation of protein was observed.

It should be emphasized that Dowex resin chromatography was the only effective method for removal of adenine nucleotides from F₁, which did not cause large, immediate, and irreversible losses in enzyme activity. Chromatography on a column of Sephadex G-50 was not always effective in removing adenine nucleotides, as judged by the ratio of absorbance at 280 nm to that at 260 nm, and the emerging enzyme preparation uniformly retained less than 70% of the initial specific activity.

Submitochondrial particles ETPH(Mg++) were prepared as described by Beyer (8).

Preparation of Aurovertin Solutions—Crystalline aurovertin was dissolved in methanol at a final concentration of about 220 μM. The exact concentration of diluted stock solutions in methanol was determined from the absorbance at 367 nm with the use of a molar extinction coefficient at this wavelength of 42,700 (9). A molecular weight for aurovertin of 490 was calculated from the molecular formula of Baldwin et al. (9). The molecular weight of 476 measured by Beechey et al. (10) is correct for the preparation used in this study, the binding data presented here would require a small correction. Aurovertin exhibited a single fluorescent spot when chromatographed, at room temperature, on Whatman No. 1 paper in either water saturated with chloroform (Rₚ = 0.68) or in a solvent consisting of water-dioxane (10:1, v/v). The Rₚ in the latter solvent was 0.82. Aliquots of a few microliters were added to reaction mixtures directly from the stock solution of aurovertin in methanol. No effect of methanol alone was observed on either fluorescence or enzyme activities under these conditions.

EXPERIMENTAL TECHNIQUE

Enzyme Assays—The ATPase activity of F₁ was determined in the presence of a regenerating system for ATP either by colorimetric assay of P₁ released from ATP (11) or, during kinetic studies, by the spectrophotometric method (11). In the kinetic studies, the reaction was started by the addition of MgATP from an equimolar solution of Mg⁺⁺ and ATP. The MgATP stock solution was freed of ADP by incubation for 15 min with 80 μmoles of ATP, pH 7.4, 80 μmoles of MgSO₄, 2 μmoles of phosphoenolpyruvate, pH 7.4, and 100 μg of pyruvate kinase in a final volume of 2 ml. The pH was unchanged at the end of the reaction. The final concentration of MgATP was 0.04 M. The stock solution contained a small amount of pyruvate which caused a small initial jump in the curve representing ATPase activity, but this did not interfere with accurate measurement of initial rates. All kinetic measurements were carried out at 25° in a Perkin-Elmer model 356 dual wave length spectrophotometer. The oxidation of NADH was monitored by recording the difference in absorbance at 340 versus 374 nm. Other conditions of each experiment are given in the legends to figures and tables. In separate experiments, it was established that aurovertin did not inhibit either pyruvate kinase or lactate dehydrogenase activity.

Fluorometric Measurements—Fluorescence emission spectra were obtained in the instruments described previously (12). Aurovertin binding measurements were carried out in either of two instruments as described (12). The filters used to isolate specific portions of the spectrum are mentioned in the legends to figures and tables. Fluorescence polarization measurements were made with a modified version of the spectrofluorometer described earlier (12). Right angle optics were used and filters replaced the emission monochromator. Both polarizer and analyzer consisted of formula 105 UV polarizing filters (Polacoat Incorporated, Cincinnati, Ohio). The polarizer was mounted between the excitation monochromator and the excitation cutoff filter (CS-7-60, Corning Glass Works). The polarized fluorescent light passed through a 2-mm column of 2 N NaN₃ and then through a Corning Glass CS-3-71 filter before reaching the analyzer. Polarization measurements with submitochondrial particles included in addition a Wratten 34A filter on the emission side of the instrument. Measurement of polarization was made by manual rotation of the analyzer and polarizer, and the data were corrected for instrumental error as described by Chen and Bowman (13). The uncertainty in the polarization measurements was within ±0.003 unit. The temperature of the sample cell was controlled by circulation of water from a thermostatted water bath through channels in the sample holder. The temperature of the cell contents was monitored with a small thermistor probe immersed in the liquid column to a point just above the exciting light beam.

The energized fluorescence response of aurovertin in the presence of ETPH was measured as described earlier for other fluorescent probes (12). The filter fluorometer with front surface optics was used with filters given in the legends to appropriate figures.

Measurement of absorption spectra were carried out in a Cary model 14 spectrophotometer fitted with the 0 to 0.1 absorbance slide-wire. The possibility that fluorescent light emitted by the sample might be sensed by the photomultiplier tube was minimized because of the distance of the cuvette from the phototube and the use of low concentrations of aurovertin.

Measurement of Protein Concentration and Definition of Units—The concentration of mitochondrial proteins was measured by a modified biuret reaction (11). The concentration of solutions of F₁ was determined by refractometry (14). One unit of ATPase activity is defined as that amount of enzyme which catalyzes the turnover of 1 μmole of substrate per min under the specified conditions of assay. Specific activity is expressed as units per mg of protein. The fluorescence increment is defined as the fluorescent intensity, in arbitrary units, when 1 μmole of aurovertin is bound to F₁.

RESULTS

The absorption spectrum of aurovertin in Buffer I is shown in Fig. 1A, Curve 1. It may be seen that the compound exhibited a maximum at 270 nm and a somewhat broader absorption band of slightly lower amplitude at 365 nm. The position of the maxima are in good agreement with those reported by Baldwin et al. (9); however, the absorption at 270 nm is about 20% lower than that at 365 nm when aurovertin is dissolved in ethanol
The nature of the interaction of aurovertin with F1, monitored by changes in fluorescence intensity, was complex and influenced by adenine nucleotides and Mg++. In the presence of Buffer I containing ATP, about 80% of the final maximum fluorescence was achieved within the first 4 s after the addition of aurovertin (Fig. 2, Curve C). However, when ATP was replaced by ADP, only about 25% of the final maximum fluorescence was reached during the same time period (Fig. 2, Curve B). In the absence of adenine nucleotides, the rate of the reaction was slower (Fig. 2, Curve A). No further changes in fluorescence intensity were observed 5 min after the addition of aurovertin.

A second series of experiments was carried out to test the effects of addition of adenine nucleotides or Mg++ on the fluorescence intensity of the aurovertin-F1 complex. The first arrows in the curves of Fig. 3 indicate the addition of 1.1 μM aurovertin to a solution of F1 in Buffer I. About 2 min later, when the intensity of fluorescence had reached a plateau, 1.6 mM ATP was added (Curve A). The small transient increase in fluorescence was followed by a slower quenching which reached a new plateau in about 2 to 3 min. Similar observations were reported by Lin (15). In separate experiments, not described here, it was found that the fluorescence emission maxima of the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B). The quenching by ATP was almost completely reversed by subsequent addition of ADP (Fig. 3). Addition of ADP to the aurovertin-F1 complex in Buffer I containing ATP occurred at the same wave lengths as those shown in Buffer I alone (cf. Fig. 1B).
3.0 ml of Buffer I. At the

reaction mixture contained 1.5
nmoles of ATP or ADP were not reversed by EDTA. The apparent
metal-free preparations of ATP were used. Moreover, the effects
of Mg++ and EDTA. The apparent
competitive interaction between ATP and ADP may be a re-

flexion of the fact that ATP can displace bound ADP from the
enzyme (17). ITP, which is a substrate for Fr, and IDP which
does not inhibit the enzyme (17), induced changes in the fluores-
cence intensity of the aurovertin-F1 complex. The enzyme was
prepared in Buffer I as described under "Materials and Methods." 

The reaction mixture for Curves A and B contained 1.15 \mu M F_1 in
3.0 ml of Buffer I. At the first arrows, 3.3 nmol of aurovertin
were added. At the second set of arrows, 4.8 \mu M of ATP (Curve
A) and 2.7 \mu M of ADP (Curve B) were added. In Curve C,
the reaction mixture contained 1.5 \mu M F_1 in 3.0 ml of Buffer 1.
After addition of 2.2 nmol of aurovertin (first arrow), 9 \mu M
of MgSO_4 were added (second arrow). The third set of arrows
marks the addition of: Curve A, 2.7 nmol of ADP; Curve B, 10
nmol of EDTA; Curve C, 4.8 \mu M of ATP. Measurement of
fluorescence and additions to the cuvette were made as described
in the legend to Fig. 2.

ATP-induced quenching such as that shown in Fig. 3 did not
appear to be due to the presence in the ATP preparations of
divalent metal ions (16), since similar effects were observed when
metal-free preparations of ATP were used. Moreover, the effects
of ATP or ADP were not reversed by EDTA. The apparent
competitive interaction between ATP and ADP may be a re-

deflection of the fact that ATP can displace bound ADP from the
enzyme (17). ITP, which is a substrate for Fr, and IDP which
does not inhibit the enzyme (17), induced changes in the fluores-
cence of the aurovertin-F1 complex which were similar to those
caused by adenine nucleotides although higher concentrations
were required. The magnitude of the effect was dependent on
the ratio of aurovertin to F_1 in the solution. ATP quenching
was maximal at low aurovertin to F_1 ratios while ADP enhance-
ment was minimal under these conditions (Table I). The brief
enhancement of fluorescence which immediately preceded the
quenching induced by ATP addition (Fig. 3) was proportional
to the measured content of ADP in the ATP preparations used
in these experiments. The amount of ADP (0.06 \mu M) added
with the usual addition of 4.8 \mu M of ATP would have been
even to account for the observed enhancement. Prior incuba-
tion of the ATP preparations with phosphoenolpyruvate and
pyruvate kinase, which converted all free ADP to ATP, elimi-
nated this phenomenon.

Binding of Aurovertin to F_1—Titrations of F_1 with aurovertin
were carried out as described under "Materials and Methods." 

A typical plot of the reciprocal of the observed fluorescence
versus the reciprocal of the protein concentration is shown in
Fig. 4. Extrapolation to infinite protein concentration of a line
fitted to the points by least squares analysis yielded the recip-
\al of the fluorescence intensity expected when all of the auro-
vertin present in solution was bound to F_1. The fluorescence
increment is defined as the extrapolated fluorescence intensity
divided by the nanomoles of aurovertin present. It may be
seen that the fluorescence increment when all aurovertin was
bound would be 184 arbitrary units in Buffer I alone and slightly
higher (198) in Buffer I containing ADP. However, quenching
was observed in Buffer I containing ATP (120) or Mg++ (56)
(not shown). Values of fluorescence intensity obtained in this
way were used to calculate the binding ratios of aurovertin to
F_1 in the presence and absence of adenine nucleotides. Scatchard
type (18) plots of the data are shown in Fig. 5. Two binding
sites per molecule of F_1 were found when the titration was carried
out in the presence of ATP (Plot A). However, in the presence
of ADP (Plot C) or in Buffer I alone (Plot D) or in the presence
of Buffer I containing Mg++ (not shown), only one binding site
for aurovertin was found per molecule of F_1. Calculation of the
dissociation constant of the aurovertin-F_1 complex, from the
data shown in Fig. 5, gave values of 0.52 \mu M and 0.07 \mu M for
titrations carried out in Buffer I containing ATP and ADP,
respectively. The dissociation constant was 0.04 \mu M for titra-

tions carried out in the Buffer I alone and 0.013 \mu M in Buffer I

**Table I**

Effect of adenine nucleotides and ratio of aurovertin to F_1 on fluores-
cence intensity of aurovertin-F_1 complex

Fluorescence intensity was measured as described in the legend
to Fig. 2. F_1, in amounts shown, was prepared in Buffer I.
Further additions were made after the fluorescence of the auro-
vertin-F_1 complex had stabilized (about 3 min). Percentage of
quenching was obtained by multiplying 100 times the absolute
difference between the fluorescence intensities before and after
addition of nucleotides divided by the fluorescence before the
addition of nucleotides.

| Aurovertin/F_1 | ATP | ADP | Quenching |
|--------------|-----|-----|-----------|
| nmol/F_1     | nmol | nmol | %         |
| 3.30         | 0.55 | 6.03 | 1.6       | 22.7 |
| 3.32         | 1.07 | 3.10 | 1.0       | 40.4 |
| 1.32         | 0.98 | 1.35 | 1.6       | 67.8 |
| 0.555        | 0.92 | 0.64 | 2.67      | 60.5 |
| 0.22         | 1.10 | 0.20 | 2.67      | 68.1 |
| 0.555        | 0.229| 2.55 | 0.9       | 40.0 |
| 0.558        | 0.534| 1.70 | 0.9       | 35.0 |
| 0.555        | 0.488| 1.27 | 0.9       | 34.5 |
| 0.558        | 0.687| 0.85 | 0.9       | 26.3 |
| 0.88         | 4.38 | 0.20 | 0.9       | 21.3 |

**Fig. 4.** The fluorescence intensity of aurovertin completely
bound to F_1. Each point on the graph represents a separate
experiment in which 0.36 nmole of aurovertin was added to the
amount of enzyme protein shown. The value of the fluorescence
intensity was recorded after the unchanging plateau value was
reached as in Fig. 3. The reciprocal of the fluorescence intensity
was then plotted versus the reciprocal of the protein concentration.
The resulting data were treated by the method of least squares
and plotted as shown. The buffers used were Curve A, buffer I;
Curve B, Buffer I containing 4 mM ATP; Curve C, Buffer I con-
taining 1 mM ADP.
Fig. 5. Binding of aurovertin to F₁. A, F₁ (0.157 μM) was titrated in Buffer I containing 4 μM ATP in a volume of 3.0 ml. Aurovertin was added stepwise to a final concentration of 16.5 μM. B, correspondence of the experimentally determined binding of aurovertin (○) with the theoretical curve (——) obtained as described in the text. The points shown represent three experiments under conditions described in Plot A with 0.077, 0.103, and 0.157 μM F₁. N, the average number of molecules of aurovertin bound per molecule of F₁, A, the concentration of free aurovertin. For calculation of the theoretical curve, Kₐ = 1.02 μM⁻¹ and n = 2.0. C, Buffer I containing 1 mM ADP. The points represent three experiments with 0.51, 0.78, and 1.03 μM F₁, respectively. The aurovertin concentration at maximum was 0.605 μM. D, titration of 1.44 and 2.17 μM F₁ in Buffer I. The aurovertin concentration ranged up to 0.279 μM. The line drawn through the points shown in A, C, and D was obtained from a least squares analysis of the data. The intercept of the line with the abscissa gives n, the total number of binding sites per mole of F₁. The intercept with the ordinate gives nKₐ.

containing Mg²⁺. Lardy and Lin (5) also have observed a low dissociation constant for the aurovertin-F₁ complex.

In order to test the accuracy of the binding data, the experimentally determined binding ratios from several titrations were plotted versus the log of the free aurovertin concentration and compared to a curve fitted to the equation (18)

\[ n = \frac{nKₐC}{1 + KₐC} \]

where Kₐ is the dissociation constant for the formation of the inhibitor-F₁ complex, n is the average number of aurovertin molecules bound per molecule of F₁, n is the number of binding sites on each protein molecule, and C is the concentration of free aurovertin. The theoretical curve was obtained by calculating \( n \) (with the use of values of n and Kₐ obtained in Plots A, C, and D of Fig. 5) at different concentrations, C, of free aurovertin.

The close correspondence of the points obtained from three titrations in the presence of ATP with the theoretical curve is shown in Plot B of Fig. 5. Similar observations were made from the data for titrations with ADP and in Buffer I without additions.

The fluorescence increment and the dissociation constant calculated for the aurovertin-F₁ complex in these experiments was influenced in the manner in which the enzyme was prepared. The dissociation constant was lowest (0.015 μM) and the fluorescence increment highest when F₁ was prepared in Buffer I and ADP was added just before the titration with aurovertin was begun. If the enzyme was prepared in the presence of ADP, the dissociation constant was about 2-fold higher. However, the stoichiometry of binding was the same in both instances.

**Polarization of Fluorescence**—Further investigations into the mechanism of enhancement of fluorescence of aurovertin were carried out by measuring the polarization of fluorescence of aurovertin. It has been reported by Weber (19) that the study of polarization phenomena may provide an insight into the environment of a fluorophore, whether this be the solvent surrounding a molecule free in solution or a protein to which the fluorophore may be bound. If the lifetime of the excited state is long relative to the time required for rotational diffusion (due to Brownian motion) then the resulting emitted fluorescent light will exhibit little or no polarization. However, if the fluorophore is in a viscous solvent or attached to a large macromolecule which rotates more slowly in solution, appreciable polarization of fluorescence may be observed.

A summary of some measurements of the polarization of fluorescence of aurovertin, along with computed values of the viscosity of the solvent used, is given in Table II. The polarization of the free inhibitor in methanol or in Buffer I containing ATP was appreciable, 0.294 and 0.278, respectively. However, the observed polarization was increased considerably in solvents of high viscosity such as 2 M sucrose or 100% glycerol at low temperatures. The polarization values recorded were affected little if at all over the range of aurovertin concentration tested. A considerable increase in polarization also was detected when aurovertin was bound to F₁. The average value of 0.375 at 25°C was similar to that observed in solvents of high viscosity.

The possibility was investigated that changes in polarization of fluorescence might accompany changes in fluorescence intensity following the addition of adenine nucleotides or Mg²⁺. The results shown in Table III indicate that both ATP and ADP caused a small increase in polarization whereas addition of Mg²⁺ caused a decrease. The magnitude of the observed changes was 3 to 8 times greater than the uncertainty in the measurements (±0.003). In each case measurements were made immediately before and after the additions shown, and the effects were observed repeatedly with a number of different enzyme preparations.

**Interaction of Aurovertin with Submitochondrial Particles**—As
shown in Fig. 6, addition of aurovertin to a suspension of ETPH resulted in a large fluorescence enhancement. Subsequent addition of ATP or Mg\(^{2+}\) led to a quenching of fluorescence whereas addition of ADP caused an enhancement of fluorescence. The quenching of ATP was reversed by addition of ADP and the observed enhancement by ADP was reversed by addition of ATP in a manner similar to that described for soluble F\(_{1}\) (Fig. 3). Although the responses to adenine nucleotides and Mg\(^{2+}\) are thus qualitatively similar to that of the aurovertin-F\(_{1}\) complex, the rate of the response was more rapid for the soluble enzyme (cf. Fig. 3).

It should be pointed out that quenching by Mg\(^{2+}\) appeared to depend on the Mg\(^{2+}\) content of the particles. It was necessary to preincubate the particles with a chelating agent for 10 min before addition of aurovertin in order to demonstrate clearly

| Experiment | Additions | \(\rho\) |
|------------|-----------|--------|
| 1          | None      | 0.313  |
| 2          | ATP       | 0.329  |
| 3          | None      | 0.321  |
| 4          | ADP       | 0.331  |
| 5          | Mg\(^{2+}\) | 0.344  |
| 6          | Mg\(^{2+}\) | 0.330  |
| 7          | Mg\(^{2+}\) | 0.315  |
| 8          | Mg\(^{2+}\) | 0.329  |
| 9          | Mg\(^{2+}\) | 0.315  |

**Effect of adenine nucleotides on fluorescence polarization of aurovertin-F\(_{1}\) complex**

Measurements of the polarization of fluorescence and preparation of F\(_{1}\) in Buffer I are described under “Materials and Methods.” Measurements were made at 26°C in a final volume of 3.0 ml. The concentration of F\(_{1}\) was 0.576 mg/ml and that of aurovertin was 0.293 mg/ml. Where indicated the concentration of ATP was 3.3 mM; ADP, 1.5 mM; MgSO\(_{4}\), 4 mM. In Experiments 4 and 5, F\(_{1}\) was dissolved in Buffer I containing ATP and ADP, respectively.

![Graph showing interaction of aurovertin with submitochondrial particles](image)

**FIG. 6. Interaction of aurovertin with submitochondrial particles.** ETPH was prepared as described under “Materials and Methods.” The particles were suspended in 3.0 ml of Buffer I at a concentration of 0.37 mg per ml and incubated for 10 min. A large increase in fluorescence followed the addition of 0.85 nmole of aurovertin (first arrow). After the increase in fluorescence intensity had slowed or stopped, 3.3 mM ATP, 4 mM MgSO\(_{4}\), or 1.5 mM ADP was added as shown. Conditions of the measurement were the same as used in Fig. 2 except that the trace was interrupted when additions were made to the cuvette.

**Effect of Energization on Fluorescence of Aurovertin**—The addition of succinate to an incubation mixture containing ETPH and aurovertin led to an enhancement of fluorescence (Fig. 8, Curve A). Energy conservation apparently was not affected by the concentrations of aurovertin used since the energized fluorescence of 1-anilinonaphthalene-8-sulfonate (12, 22) was not influenced under these conditions. Quenching of the fluorescence of aurovertin followed the onset of anaerobiosis (Fig. 8, Curves A and B) or the addition of carboxyl cyanide p-trifluoromethoxyphenyldiazoxide (not shown). Whereas the energized fluorescence response of 1-anilinonaphthalene-8-sulfonate was enhanced by rutamycin (22), that of aurovertin was prevented by prior incubation with rutamycin or carboxyl cyanide \(\beta\)-trifluoromethoxyphenyldiazoxide (Fig. 8, Curve C). A quantitative difference in aurovertin fluorescence was observed when EDTA replaced Mg\(^{2+}\) in the reaction mixture (Curve B). It may be seen that in this case both the fluorescence enhancement following the addition of aurovertin and the response to succinate was markedly increased in the presence of EDTA. Moreover, only a small quenching was observed following anaerobiosis.

**Inhibition of ATPase Activity by Aurovertin**—In order to correlate binding of aurovertin with inhibition, the time course of ATPase activity was compared with the time course of the development of fluorescence as illustrated in Fig. 2. In Fig. 9 Curve A is a control showing the rate of ATP hydrolysis. In the experiment illustrated by Curves B and C, 0.22 and 0.66

**FIG. 7. Polarization of fluorescence of the aurovertin-ETPH complex.** ETPH in the amounts shown was added to a reaction mixture (final volume 3.0 ml) which contained Buffer I, 4 mM ATP, and 0.392 mg aurovertin. Polarization was measured as described under “Materials and Methods,” at 22°C. The reciprocal of the polarization measured at each concentration of protein is plotted versus the concentration.
ANAEROBIOSIS

Fig. 8. Energized fluorescence response of aurovertin. Fluorescence measurements were made in the filter fluorometer with front surface optics and filters described in the legend to Fig. 2. In addition, a Wratten number 55 filter was placed in the emitted fluorescent light path. The reaction mixture contained, in a final volume of 3.0 ml, 1.2 mg of ETPH and 20 mM potassium phosphate buffer, pH 8.0. The experiment represented by Curve A contained in addition 2 mM MgSO₄, while that of Curve B contained 2 mM EDTA. In Curve C, the particles were pretreated with either 8 μg of rutamycin or 2 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone. At the first arrows (Point 1), 2.2 nmoles of aurovertin were added. When the fluorescent signal reached a final value, the position of the recorder pen was readjusted with the zero control to establish a new base-line (Point 2). Succinate (8.3 μM) was added as shown.

nmole of aurovertin, respectively, were added to the reaction mixture 15 s after the addition of substrate. It may be seen that there was a lag in the onset of inhibition unless the inhibitor was incubated with F₁ for 2 min prior to the addition of substrate. Lineweaver-Burk plots (23) indicated that the inhibition was uncompetitive with ATP (Fig. 10). Whereas the inhibition by aurovertin of the ATPase activity of submitochondrial particles was reported to be different in Tris versus phosphate buffer (24), the inhibition curves obtained with the soluble enzyme were the same in either buffer.

DISCUSSION

A variety of observations suggest that the conformation of F₁ in solution may be modified by interaction with adenine nucleotides or Mg²⁺. Thus these substances alter the fluorescence intensity, the dissociation constant, and the polarization of fluorescence of the aurovertin-F₁ complex. Direct evidence for an ATP-induced structural change in F₁ was obtained from measurement of intrinsic viscosity (12) and the polarization of fluorescence of pyrenebutyric acid covalently linked to the enzyme. The rotational relaxation time of the conjugate was 750 ns in Buffer I which contained ATP and 2000 ns in Buffer 1 alone.

As prepared in this study, the ATPase activity of the Dowex-treated enzyme preparations remained stable at room temperature for about 5 hours. The subsequent losses in activity were accompanied by the development of turbidity and, at high concentrations (5 to 10 mg per ml), precipitation of denatured protein. These time-dependent changes in the physical state of the nucleotide-free enzyme may help to explain the variations observed in the polarization of fluorescence of the aurovertin-F₁ complex in Buffer 1 as well as the finding that addition of ATP to the inhibitor-F₁ complex in Buffer I frequently did not restore the polarization to values observed with enzyme samples which were prepared in the presence of ATP. It would appear, therefore, that the effectiveness of ATP and ADP in restoring the original conformation of F₁ decreased with the age of the preparation.

Fig. 9. Inhibition of ATPase activity of F₁ by aurovertin. ATPase was measured by a spectrophotometric method (11) as described under “Materials and Methods.” The reaction mixture contained in a final volume of 2.85 ml, 30 μmoles of Tris sulfate, pH 8.0, 6 μmoles of phosphoenolpyruvate, 0.68 μmole of DPNH, 50 μg of pyruvate kinase, 25 μg of lactate dehydrogenase, and 1.4 μg of F₁. The reaction was started by adding 50 μl of a solution containing 0.04 mM MgATP (first arrows). Curve A, ATP hydrolysis without inhibitor. Aurovertin was added at the second arrow; 0.22 nmoles in Curve B and 0.66 nmoles in Curve C. Curve D, 0.66 nmoles of aurovertin was incubated with F₁ in the reaction mixture for 2 min prior to the addition of MgATP. The trace was interrupted for addition of materials to the cuvette.
The changes in structure which did occur in the enzyme following removal or addition of adenine nucleotides were not accompanied by significant uncoiling of the protein since estimates of the molar ellipticity of the enzyme, based on measurements of circular dichroism at 220 nm, were identical in Buffer I alone and in Buffer I which contained adenine nucleotides or MgSO₄.

Mechanism of Enhancement and Quenching of Fluorescence of Aurovertin—It has been observed that a variety of dyes such as 1-anilino napthalene-8-sulfonate and 2-p-toluidinaphthalene-6-sulfonate exhibit little or no fluorescence in water but fluoresce strongly when dissolved in nonpolar solvents. When bound to proteins, aurovertin is strongly quenched in nonpolar solvents and in viscous solvents such as sucrose. Direct interaction of aurovertin with submitochondrial particles was consistent with the findings of Chance et al. (32) that the enzyme-binding site. Chance et al. (32) have reported no change in the fluorescence intensity of aurovertin bound to submitochondrial particles that occurred when aurovertin bound to the mitochondrial membrane may possibly be more readily interpreted than those of luciferase and anilinonaphthalene-8-sulfonate and other dyes since it seems unlikely that the enhanced fluorescence of the aurovertin-F₁ complex was a reflection of "polarity" effects since the inhibitor exhibited about the same low fluorescence in nonpolar solvents as in aqueous buffers. It seems more likely that the fluorescence of the aurovertin-F₁ complex reflected an increased rigidity imposed upon the fluorophore in the protein-binding site. This conclusion is supported by the finding that aurovertin fluorescence (Fig. 1) and its fluorescence polarization (Table II) were enhanced considerably in solutions of high viscosity. The increased polarization of fluorescence of bound versus free aurovertin, 0.375 and 0.279, respectively, strengthens the suggestion of reduced mobility in the enzyme-binding site. Thus a reduction of collisional quenching by solvent molecules would appear to make an important contribution to the fluorescence enhancement of aurovertin bound to F₁.

An alternate explanation is suggested by the work of Oster and Nishijima (28) who observed an increased quantum yield of fluorescence of auramine O, a substituted diphenylmethane dye, when the compound was dissolved in solutions of high viscosity. The authors' interpretation of their data, that the fluorescence intensity of auramine O depended on the rotational diffusion constant of the rotating phenyl groups of the molecule, might equally well apply to aurovertin if this compound also contained light emitting groups capable of internal rotation.

The quenching of fluorescence brought about by addition of ATP to the aurovertin-F₁ complex was, at least in some instances, partly due to the release of aurovertin from the enzyme. It may be calculated from Table I, line 5, that 0.194 nmole of aurovertin was bound to F₁ in the absence and 0.127 nmole in the presence of ATP. In this case the molar ratio of aurovertin to F₁ was 0.2. When the molar ratio was 6 (Table I, line 1), 0.5 nmole was bound in the absence and 0.69 nmole in the presence of ATP. However, most of the fluorescence quenching by ATP may be ascribed to the decrease in the fluorescence increment (Fig. 4). The enhancement of fluorescence by ADP may be explained by the observation that the fluorescence increment increased without change in the stoichiometry of binding. Similarly, the remarkable quenching by Mg⁺⁺, which also occurred in the absence of a change in the amount of aurovertin bound to F₁, appeared to be due to a 3-fold decrease in the fluorescence increment. In separate experiments, not shown here, it was found that Mg⁺⁺ did not quench the fluorescence of aurovertin in viscous solvents such as 2 M sucrose. Direct interaction of Mg⁺⁺ with aurovertin on the enzyme would thus seem to be an unlikely explanation of the quenching. However, it is not ruled out that such an interaction may be promoted in the enzyme-binding site.

It seems reasonable to conclude that the conformational changes which occur in F₁ alter the aurovertin-binding sites and give rise to the observed changes in fluorescence. Thus aurovertin would appear to be a sensitive probe of conformational changes in the enzyme.

Mechanism of Inhibition of ATPase Activity by Aurovertin—The finding that aurovertin inhibition of ATP hydrolysis catalyzed by F₁ was uncompetitive agreed with previous observations of Lin (15). Lardy et al. (1) also found that the inhibition of mitochondrial ATPase by aurovertin was uncompetitive. Although two binding sites for aurovertin were available on F₁ in the presence of ATP (Fig. 5A), only one of these sites appeared to be the inhibitory site since the reciprocal of the inhibited rate of the ATPase was linearly related to the aurovertin concentration. Although other interpretations are possible, it may be expected that a linear relation would be obtained only with the square of the aurovertin concentration if inhibition required occupation of both sites (29). It is not entirely clear which of the two sites might be inhibitory. The experiment illustrated in Fig. 9 suggested that the single site available for aurovertin in Buffer I was the inhibitory site since preincubation of the enzyme with aurovertin prior to the addition of substrate led to an inhibited initial reaction rate whereas a lag was observed in the onset of inhibition when aurovertin was added to an ongoing reaction. This lag appeared to be of about the same order of magnitude, 10 s, as the time required for formation of the aurovertin-F₁ fluorescent complex in Buffer I containing ATP. However, a rapid rearrangement of bound aurovertin to an inhibitory site upon the addition of ATP cannot at present be ruled out. The possibility that the two binding sites for aurovertin on F₁ only one site participates in the inhibition of ATPase activity may serve to explain observations that the forward reactions of oxidative phosphorylation (for example, ATP synthesis) are much more sensitive to aurovertin inhibition than the reverse reactions such as the ATP-supported reduction of DPN⁺ by succinate and the ATPase of submitochondrial particles (2, 3). It may be that the second binding site for aurovertin on F₁ participates more directly in the inhibition of forward reactions of oxidative phosphorylation. The possibility of two apparently unidirectional catalytic sites on F₁ is consistent with the findings of Asani et al. (30) that the protein inhibitor of F₁ (31) inhibited the reverse reactions of oxidative phosphorylation but, as shown by Pullman and Monroy (31), did not inhibit the forward reaction.

Interaction of Aurovertin with Submitochondrial Particles—The fluorescence responses of aurovertin on the mitochondrial membrane may possibly be more readily interpreted than those of 1-anilino napthalene-8-sulfonate and other dyes since it seems probable that aurovertin is localized exclusively on F₁. Lin has noted little enhancement of fluorescence in the presence of submitochondrial particles treated so as to remove most or all of the membrane-bound F₁. (15). Lardy and Lin (5) have noted that F₁ was the only protein in mitochondria that enhanced the fluorescence of aurovertin. It was also reported in this paper that ATP, ADP, and Mg⁺⁺ caused changes in the fluorescence intensity of aurovertin bound to ETPH which were similar to those with F₁. Thus the available evidence supports the suggestion that aurovertin forms a specific complex with F₁ on the mitochondrial membrane. The inhibition by rutamycin of the succinate-induced energized response of aurovertin was consistent with the suggestion of Lee and Enser (3) and Robertson et al. (4) that aurovertin acts on the ATP side of the rutamycin-sensitive site. Chance et al. (32) have reported no change in the fluorescence intensity of aurovertin bound to submitochondrial particles that was related to turnover of the respiratory chain. However, since these experiments were carried out in the presence of rutamycin, it appears likely that the enhancements observed were due to a conformational change in the enzyme.
mycin, energized responses of the kind reported in this paper would not have been observed.

The changes in the fluorescence intensity of the aurovertin-ETPH complex which accompany the appearance of the energized state may well reflect an altered conformational state of membrane-bound F1 in a manner analogous to the alterations suggested above for the soluble F1-inhibitor complex. The ambiguities introduced into the measurement of polarization by scattered light (33), which was considerable in suspensions of submitochondrial particles (Fig. 7) precluded, in our view, a search for the effects of adenine nucleotides or Mg++ on the polarization of fluorescence of the membrane-bound complex which might parallel the effects observed with soluble F1. It may well be, however, that measurement of pulsed anisotropy of emission (34, 35) will provide a useful approach to this type of study of conformational change in F1 on the mitochondrial membrane.

That a change in the conformation of a coupling factor may occur during the energized state is suggested by the tritium exchange experiments of Boyer and Jagendorf (36) with the coupling factor from spinach chloroplasts. In addition, the suggestions of Boyer (37) and the work of Hackenbrock (38), Green and Ji (39) and others, predict conformational changes in the mitochondrial membrane in the energized state. It was pointed out (charge experiments of Ryrie and Jagendorf (36) with the coupling factor from spinach chloroplasts. In addition, the suggestions of Boyer (37) and the work of Hackenbrock (38), Green and Ji (39) and others, predict conformational changes in the mitochondrial membrane in the energized state. It was pointed out above, however, that at least part of the observed fluorescence quenching, following for example the addition of ATP, may be ascribed to dissociation of the fluorescent aurovertin-F1 complex. Moreover, the experiment illustrated in Fig. 8 suggests that Mg++ is intimately involved in the energized fluorescence response of aurovertin on submitochondrial particles. It may be that Mg++ is released from membrane-bound F1 during energization, thus permitting an enhancement of fluorescence by the mechanism suggested above. Following anaerobiosis, Mg++ reattaches to F1 if the metal is available in solution and quenching ensues. If Mg++ is not available, for example in the presence of a chelator, little quenching occurs.

Based upon studies carried out with soluble F1, the changes in aurovertin fluorescence during energization of ETPH would thus appear to be due to a change in conformation of the bound enzyme which alters the aurovertin-binding sites on the molecule. The resulting alterations might then cause quenching or enhancement as discussed above, or alternatively, might promote dissociation of the fluorescent complex.

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