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INTERACTION OF BEEF-HEART MITOCHONDRIAL ATPase, COUPLING FACTOR F_1, WITH AUROVERTIN

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SUMMARY

The antibiotic aurovertin binds to beef-heart mitochondrial ATPase, coupling Factor F_1, with biphasic fluorescence enhancement. Specific binding effects, polarity and viscosity changes may all contribute to the enhancement. Evidence is presented that it stems from aurovertin binding followed by a slow conformational change in F_1. This occurs more rapidly in dissociated F_1. The effect of substrates of the enzyme on the fluorescence enhancement is examined. Evidence is presented that in the absence of added magnesium, F_1 can hydrolyse low concentrations of added ATP.

INTRODUCTION

Beef-heart mitochondrial ATPase, coupling Factor F_1, has a mol. wt of about 350 000 and contains 5 different classes of subunits, which have been separated and chemically characterized [1–3]. The dissociation of F_1 on dilution has been detected by ultracentrifugation [4, 5] and polyacrylamide-gel electrophoresis [1].

The ATPase activity of F_1 is activated by divalent metal ions [6, 7], and inhibited competitively by ADP [8]. The other product, phosphate, has no effect.

The antibiotic aurovertin is an effective inhibitor of oxidative phosphorylation, probably because of its interaction with F_1 [9]. It is less effective as an inhibitor of ATP-driven reactions, including the ATPase activity of F_1 [10].

This report describes experiments on the formation and properties of the fluorescent aurovertin–F_1 complex, previously studied by Lardy and Lin [9] and Bertina et al. [11, 12].

MATERIALS AND METHODS

F_1 was prepared according to the method of Datta and Penefsky [13] and stored in 0.25 M sucrose, 10 mM Tris–acetate, 2 mM EDTA and 4 mM ATP at pH

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7.5. Prior to use the enzyme was centrifuged down, washed twice with an ice-cold mixture of 1 vol. saturated ammonium sulphate solution and 1 vol. 25 mM Tris–acetate buffer (pH 7.5) and dissolved in the Tris–acetate buffer. The F_1 was then shaken for 1 min with activated charcoal (2 mg per mg F_1) to remove any relatively weakly bound adenine nucleotide. The sample was then spun at 4000 rev./min for 10 min to remove the charcoal and dialysed against buffer for 2 h. Any denatured protein was spun down. A stock (3.0 μM) solution of the resulting F_1 sample lost less than 10% of its activity overnight.

Aurovertin was prepared in this laboratory as described by Bertina [11]. The fluorescence excitation spectrum (corrected for the spectrum of the fluorimeter lamp) was the same as its ultraviolet absorption spectrum. Also, the fluorescence emission spectrum observed was independent of the exciting wavelength. It follows that any impurity present in the sample was either non-fluorescent or had the same fluorescent characteristics as aurovertin. The sample used had the same biological activity as an aurovertin sample obtained as a gift from Dr H. Lardy.

Other compounds used were of the purest commercially available grades.

Aurovertin concentrations were determined using the absorbance coefficient of 42700 $M^{-1} \cdot cm^{-1}$ at 367.5 nm [14]. Protein concentrations were determined using Lowry's method [15]. ATPase activities were measured by determining the phosphate from the hydrolysis of ATP [6]. Magnesium determinations were carried out by flame photometry of F_1 samples prepared as described above.

All experiments were carried out in 25 mM Tris–acetate buffer (pH 7.5) at 25 °C. Fluorescence spectra were measured on a Perkin–Elmer fluorescence Spectrophotometer MPF-2A with a Hitachi Recorder QPD 33. Other fluorescence measurements were made with an Eppendorf fluorimeter with excitation filter 313+366 nm and emission filter 470–3000 nm. The machine had a modified sample compartment which permitted continuous automatic stirring.

Sedimentation velocity runs were carried out on an MSE analytical ultracentrifuge, fitted with Schlieren optics.

RESULTS

Aurovertin fluorescence

In the beam of the fluorimeter's lamp aurovertin fluorescence decreased to a constant level. This effect was reversible in the dark, was negligible in the presence of F_1 and could be totally removed by continuous stirring. The excitation beam caused no change in the excitation and emission peaks of aurovertin fluorescence. The spectra of aurovertin in the presence of F_1 were measured using a narrow excitation slit (4 nm) and recording the fluorescence reading reached immediately after stirring.

The fluorescence of aurovertin was enhanced on binding to F_1 by a factor of about 180 relative to water. The relative enhancements in ethanol, methanol, glycerol–water(1 : 1, v/v), diethyl ether and dioxan were 1.4, 0.9, 1.4, 0.75 and 1.7, respectively. When aurovertin bound to F_1 there was a shift from 365 nm to 373 nm in the uncorrected excitation maximum. This was not observed in any solvent investigated.

A model for the aurovertin–F_1 interaction

The fluorescence increase on adding aurovertin to F_1 is biphasic, a rapid rise

being followed by a slow phase, with a half-life between 15 and 100 s (Fig. 1). The rate of the fast phase could not be resolved under the conditions used. The rate-determining step of the slow phase could in principle be aurovertin binding, dissociation of the enzyme or a conformational change in F_1. Since no significant variation in the first-order rate constant for this slow phase was found over a 10-fold aurovertin concentration range (Fig. 2), the possibility of slow binding could be eliminated. Since saturating concentrations of aurovertin caused no disaggregation of F_1 detectable in the ultracentrifuge, it is unlikely that the slow fluorescence enhancement corresponds to enzyme dissociation. Thus, it is more probable that aurovertin fluorescence detects a slow conformational change in F_1 which follows the rapid enhancement caused by binding:

$$F_1 + \text{aurovertin} \xrightarrow{\text{fast}} F_1\text{-aurovertin} \xrightarrow{\text{slow}} F'_1\text{-aurovertin}$$

This slow change occurs more rapidly at low F_1 concentrations where the enzyme is dissociated (Fig. 3).

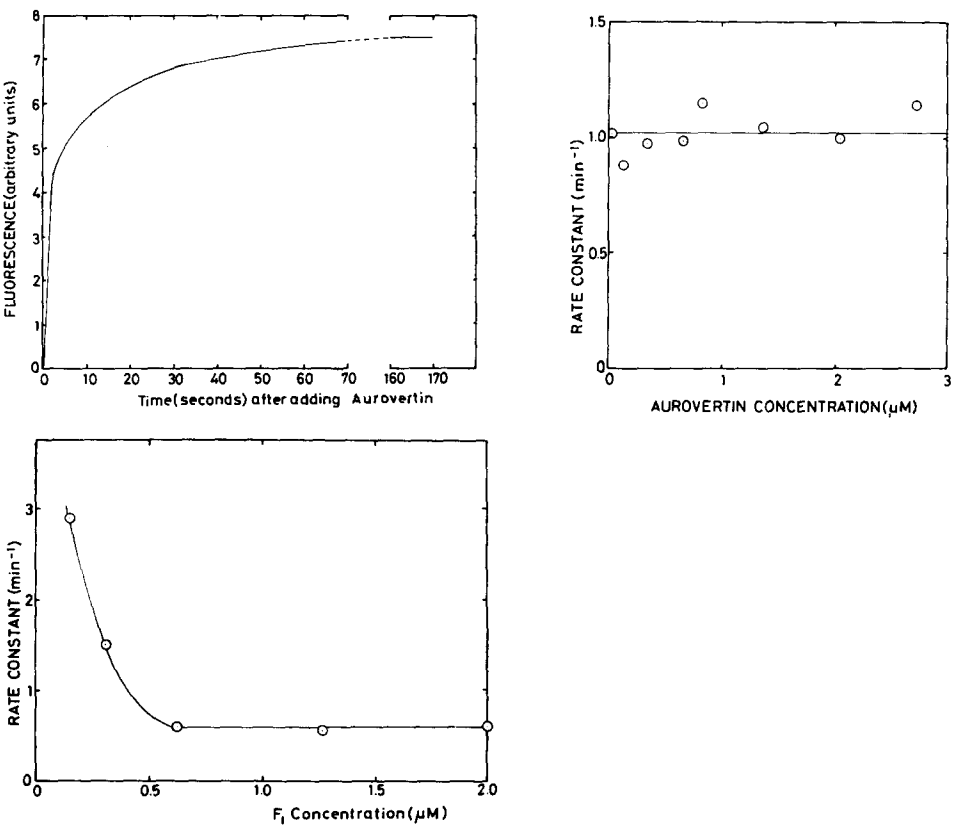

Fig. 1. Biphasic enhancement of aurovertin fluorescence by F_1. 0.2 μM F_1, 0.26 μM aurovertin.

Fig. 2. Rate of the slow enhancement of aurovertin fluorescence, at different aurovertin concentrations, 0.4 μM F_1.

Fig. 3. Rate of the slow enhancement of aurovertin fluorescence at different F_1 concentrations. 0.20 μM aurovertin.

Effect of substrate

Phosphate quenched the slow phase of the F_1-induced enhancement of aurovertin fluorescence. With 0.3 μM F_1 and 0.27 μM aurovertin, saturating phosphate concentrations reduced the slow phase to 53% of its value in the absence of phosphate. The half-concentration for this effect was 0.11 mM phosphate. There was no effect on the fast phase, nor on the rate of the slow phase.

ADP increased the extent of the slow phase of the aurovertin fluorescence enhancement and slowed it down. The half-concentration for both effects was 2–3 μM ADP (Fig. 4).

Magnesium had no effect on the extent of the fluorescence enhancement. However, saturating concentrations of magnesium increased the rate of the slow phase from 2.5 to 3.5 min^{-1}, with 0.6 μM aurovertin and 0.2 μM F_1. The half-concentration for this effect was approximately 0.3 mM magnesium.

The addition of saturating concentrations of ATP to an aurovertin–F_1 mixture quenched aurovertin fluorescence. However, after a lag of about 1 min, the fluorescence rose slowly to a constant level, equal to that attained in the presence of

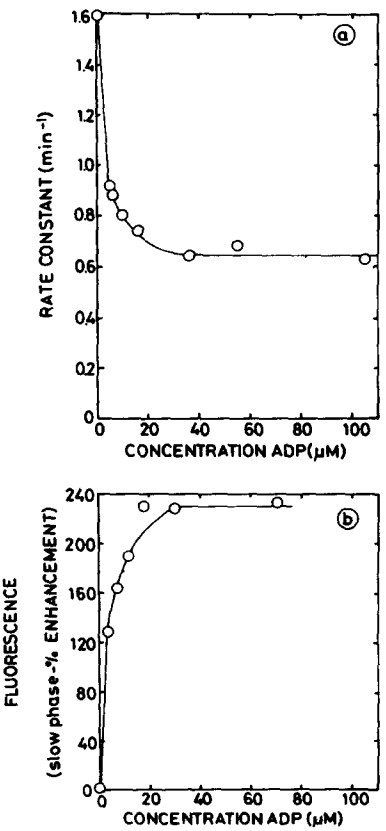

Fig. 4. (a) Deceleration and (b) enhancement by ADP of the slow phase of the aurovertin fluorescence enhancement. 0.3 μM F_1, 0.34 μM aurovertin.

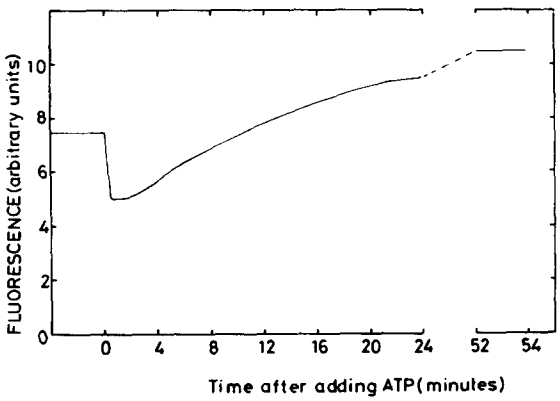

Fig. 5. Effect on aurovertin fluorescence of the addition of 9 μM ATP to 0.2 μM F_1 and 0.26 μM aurovertin.

saturating concentrations (9 μM) of ADP (Fig. 5).

When aurovertin was added 15 s after the ATP the slow part of the resulting fluorescence rise was distinctly biphasic. However, when ATP was incubated with F_1 for 10 min before adding aurovertin there was only one slow phase, of the same rate and extent as that resulting when aurovertin was added to saturating ADP (Fig. 6).

This suggests that ATP is hydrolysed by F_1 in the absence of added magnesium (even though the preparation was stored in 2 mM EDTA, 2.2 moles of magnesium were found to be present per mole of enzyme). However, determinations of P_i showed that under the conditions of this experiment less than 20 μM of 5 mM ATP was hydrolysed in 20 min.

The half concentration for the ATP-induced quenching was extremely low, approx. 0.15 μM ATP in the presence of 0.26 mM aurovertin and 0.2 μM F_1. Under these conditions the maximal fluorescence quenching was 22%.

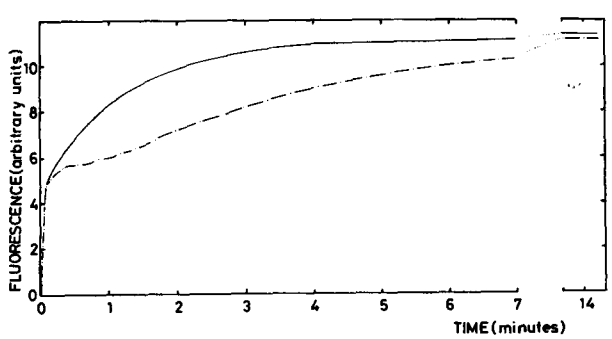

Fig. 6. Effect of pre-incubation of F_1 with ATP for 10 min (———) or 15 s (- - -) on fluorescence reached on subsequent addition of aurovertin. 0.2 μM F_1, 9 μM ATP, 0.26 μM aurovertin.

DISCUSSION

Aurovertin fluorescence is enhanced in solvents of low polarity or of high viscosity relative to water. Either or both of these factors may contribute to the fluorescence enhancement when aurovertin binds to F_1. However, the enormous size of this enhancement and the shift in the fluorescence excitation maximum from 365 to 373 nm suggest that specific binding effects are important.

Evidence has been presented that aurovertin induces a conformational change in isolated F_1 and that this change occurs more rapidly when the enzyme is dissociated on dilution. It is highly probable that aurovertin also induces a conformational change on binding to F_1 in mitochondria. Aurovertin inhibition of oxidative phosphorylation is slowly developed [11]. This may indicate that the slowly induced form is of low catalytic activity. It is possible to speculate that aurovertin reverses conformational changes in F_1 induced by the respiratory chain.

The effects of ATP, ADP, magnesium and phosphate on aurovertin fluorescence show interaction between these ligands and aurovertin on F_1. This may be direct, or involve conformational changes in the enzyme. Mitchell and Moyle [16] have shown that aurovertin reverses the phosphate-induced activation of the ATPase reaction in sonic particles. It is possible that aurovertin interacts with phosphate bound to this activating site, rather than to the catalytic site.

After the completion of this work a paper was published reporting studies by Chang and Penefsky on the fluorescence properties of the aurovertin–F_1 complex [17]. These workers found quenching of the fluorescence of F_1-bound aurovertin by magnesium. The most probable explanation of the discrepancy between those results and the work reported here is that Chang and Penefsky used F_1 which had not been treated with charcoal. The author also found magnesium induced quenching in non-charcoal treated F_1 (R. A. Yeates, unpublished observations). Chang and Penefsky did not report the reversal of the ATP-induced quenching of the fluorescence of bound aurovertin. It seems probable that under their conditions this was too slow to be readily observed.

The fluorescence results suggest that F_1 can hydrolyse small amounts of ATP in the absence of added magnesium. However, phosphate determination shows that under these conditions large amounts of ATP are not hydrolysed. One possible explanation of the results is that under these conditions strong substrate inhibition of the ATPase reaction occurs.

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