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INTERACTION OF AUROVERTIN WITH SUBMITOCHONDRIAL PARTICLES, DEFICIENT IN ATPase INHIBITOR

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SUMMARY

1. Binding of aurovertin to submitochondrial particles deficient in ATPase inhibitor is accompanied by an enhancement of the fluorescence by at least 100-fold.

2. This change in fluorescence proceeds in three phases. The slowest change may be due to a conformational change in F_1, induced by the antibiotic bound during the rapid phases, giving rise to an increase in the quantum yield of the bound fluorochrome.

3. Phosphate and ATP quench the fluorescence of the particle–aurovertin complex and ADP enhances it; the rate and extent of these changes are dependent on the availability of free Mg^{2+}.

4. There is at least one binding site on the submitochondrial particles, where ATP, ADP and phosphate can bind reversibly and for which these ligands compete. These interactions are dependent on the availability of free Mg^{2+} and are partly sensitive to oligomycin.

5. Binding studies reveal two binding sites for aurovertin on inhibitor-free particles, one with high affinity and one with a lower affinity. Ligands such as phosphate and ATP decrease both the quantum yield and the affinity of the particles for aurovertin. They also increase the total concentration of binding sites, and affect the relative contribution of weak and strong binding sites.

6. A model is presented in which changes of the aurovertin fluorescence reflect conformational changes of the ATPase induced by its ligands.

INTRODUCTION

The antibiotic aurovertin, introduced by Lardy [1] in 1961, is a potent inhibitor of oxidative phosphorylation. It also inhibits partial reactions in oxidative phos-

Abbreviations: F_1, mitochondrial ATPase; A particles, submitochondrial particles prepared by sonication of beef-heart mitochondria in an ammonia solution at pH 9.2; AS particles, submitochondrial particles prepared by treatment of A particles with Sephadex G-50; OSCP, oligomycin-sensitivity-conferring protein; I, mitochondrial ATPase inhibitor; TES, *N*-Tris(hydroxymethyl) methyl-2-aminoethanesulphonic acid.

phorylation such as the $^{32}P_i$–ATP exchange reaction and the exchange of ^{18}O between inorganic phosphate and ATP, catalysed by rat-liver mitochondria [2]. Aurovertin is, however, much less effective than oligomycin in inhibiting ATP-driven reactions in mitochondria [2–6].

Originally, Lardy et al. [2] proposed that aurovertin acts at a point between the respiratory chain and the site of action of oligomycin, but Lee and Ernster [4] and Roberton et al. [3] suggested that it acts on the ATP side of the oligomycin-sensitive site. Definite proof for the latter view came from the observation of Lardy and Lin [7] that aurovertin binds to the mitochondrial ATPase, F_1, with the stoicheiometric formation of a fluorescent complex.

Several authors [6, 8–10] have made use of the large enhancement of the fluorescence upon binding of aurovertin to F_1 to investigate the properties of both the soluble and membrane-bound enzyme. The changes of the fluorescence of the aurovertin–F_1 complex brought about by addition of ligands of the ATPase or by energizing mitochondria or submitochondrial particles are most easily interpreted in terms of conformational changes of F_1. As an example we may cite the positive co-operative binding of aurovertin to State-3 mitochondria described by Bertina and co-workers [6, 8].

Aurovertin shares the property of specific binding to F_1 with two small proteins, oligomycin-sensitivity-conferring protein (OSCP) [11, 12] and the mitochondrial ATPase inhibitor [13–15]. The aim of this and following papers is to examine the interaction between three types of ligand of mitochondrial ATPase, namely aurovertin, ATPase inhibitor and the substrates of the enzyme (ATP, ADP and P_i). This paper will deal with the reactions of the inhibitor-deficient enzyme bound to the mitochondrial membrane.

METHODS AND MATERIALS

Preparations

Heavy beef-heart mitochondria [12], A particles [16], AS particles [14] and coupling factor F_1 [17] were prepared according to the published procedures, with one slight modification for A particles. Before and after sonication the mitochondria were allowed to stand for 15 min at 4 °C in a solution containing 125 mM sucrose and 1 mM EDTA and brought to pH 9.2–9.4 with ammonia to minimize the amount of ATPase inhibitor in the particles. The specific ATPase activity of these particles was 6.5 μequiv H^+/min per mg protein. The AS particles [14], which are virtually devoid of the inhibitor, showed a specific ATPase activity of 10 μequiv H^+/min per mg protein.

Crystalline aurovertin D was isolated in pure form in this laboratory from *Calcarisporium arbuscula Preuss* (Peoria-collection A-12, 139) as described by Bertina [6]. Its physical and biological properties were essentially the same as those of a sample of aurovertin D obtained as a gift of Dr H. A. Lardy [6]. No fluorescent impurities could be detected [10]. It was dissolved in pure ethanol, and the concentration was determined spectrophotometrically at 367.5 nm using an absorbance coefficient of 42.7 $mM^{-1} \cdot cm^{-1}$ [18]. The molecular weight of aurovertin was taken to be 490 [6, 18].

Excitation and emission spectra with sufficiently concentrated aurovertin D

solutions (1–2 μM) can be obtained in aqueous solutions [8, 10]. The observed excitation maxima at 272 nm and 365 nm are close to the ultraviolet absorption maxima [10]. Using the sample provided by Dr Lardy, an emission maximum is found at about 470 nm with a shoulder near 490 nm [8, 9]. In the sample used in this investigation this shoulder was lacking [10]. When aurovertin is bound to submitochondrial particles or F_1 the fluorescence increases at least 100-fold [8–10] and the absorption maxima are shifted to 278 and 373 nm [10].

Measurement of ATPase activity

Initial rates of ATPase activity were calculated from the change of pH of the reaction medium measured with a sensitive pH-measuring system as described previously [14].

Fluorometric measurement

The interaction of aurovertin with submitochondrial particles was measured in an Eppendorf fluorimeter with excitation filter 366 nm and emission filter 430–3000 nm. The fluorimeter was equipped with a thermostatted sample compartment (made of quartz, capacity 1.5 ml) with a continuous-stirring device and front-face optics. Fluorescence yield was recorded at 30 °C in a standard medium containing 250 mM sucrose, 25 mM Tris–acetate buffer (pH 7.3) and 25 mM glucose.

Analytical methods

Soluble protein was determined by the method of Lowry et al. [19] with bovine serum albumin as a standard, insoluble protein by the biuret method as described by Cleland and Slater [20].

Materials

Hexokinase, ATP, ADP and AMP were obtained from Boehringer und Söhne. Oligomycin was kindly provided by the Upjohn Chemical Co. All other chemicals were of analytical-grade purity. Hexokinase (200 units/ml and mixed with 0.1% defatted bovine serum albumin [21]) was, before use, twice dialysed for 3 h against an excess of 10 mM Tris–acetate buffer (pH 7.3) containing 1% glucose. All organic acids used were neutralized with Tris to pH 7.3.

RESULTS

Interaction of aurovertin with AS particles

The interaction of aurovertin with AS particles, measured as the enhancement of the fluorescence, is triphasic as can be seen in Fig. 1. A very fast rise, complete within the first 2–3 s after the addition of aurovertin, precedes the fast phase (the response time of the recording system was less than 0.5 s, including mixing time). After this initial jump, a fast phase is followed by a slower phase.

The interaction between soluble F_1 and aurovertin is also at least biphasic [9, 10] and an initial jump in the fluorescence is also detected in this case (unpublished observations). Yeates [10] found that with soluble F_1 the rate of the slow phase is independent of the aurovertin concentration, but decreased more than 5-fold when the concentration of F_1 was increased from 0.15 to 0.6 μM. He interpreted these

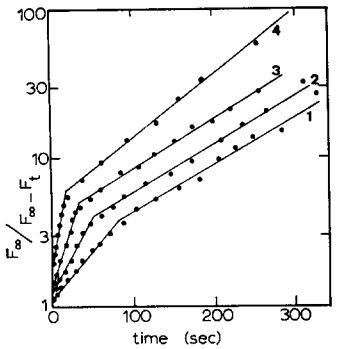

Fig. 1. Determination of first-order rate constants for the interaction of AS particles with aurovertin. Aurovertin was added to AS particles (0.15 mg protein/ml) incubated in the standard medium (see Methods and Materials). The rise in the fluorescence was recorded starting with an expanded time scale (12 cm/min). The final level reached, 5–10 min after the addition of aurovertin, is termed F_∞. The first-order rate constant can be calculated from the half time ($t_{\frac{1}{2}}$) using the formula: $k = (0.7/t_{\frac{1}{2}})\ s^{-1}$. Concentrations of aurovertin were: Curve 1, 41 nM; Curve 2, 100 nM; Curve 3, 200 nM; Curve 4, 400 nM.

results as a rapid binding of aurovertin to F_1 followed by a slow conformational change of F_1, induced by aurovertin, the latter change being more rapid in partially dissociated F_1.

Fig. 2 shows that the rate constant of the fast phase increases with the aurovertin concentration, at least at low protein concentrations. It also increases with the protein concentration at low aurovertin concentrations. This is to be expected for a bimolecular binding process.

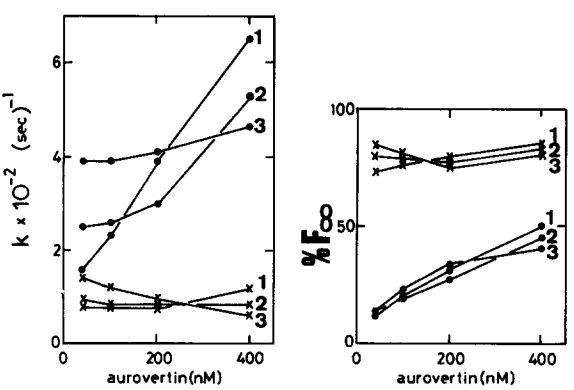

Fig. 2. Rate constants of fast and slow phases of enhancement of the fluorescence at different protein and aurovertin concentrations. Concentrations of AS particles (mg protein/ml): Curves 1, 0.15; Curves 2, 0.34; Curves 3, 0.61. ●–●, First-order rate constants for the fast phase, ×–×; first-order rate constants for the slow phase.

Fig. 3. Relative contribution of the different phases of aurovertin fluorescence enhancement at different protein and aurovertin concentrations. AS particles (mg protein/ml): Curves 1, 0.15; Curves 2, 0.34; Curves 3, 0.61. ●–●, Percentage of the final fluorescence level (F_∞), 2–3 s after the addition of aurovertin (initial jump, Fig. 1). ×–×, Percentage of F_∞ at the intersection points of the lines in Fig. 1 (fast phase).

The extent of the initial jump is also dependent on the aurovertin concentration (Fig. 3), but not on the protein concentration. This may also be explained by fast binding but presumably different from the one represented by the fast phase. The extent of the initial jump plus the fast phase is independent of the aurovertin concentration, indicating that only the relative distribution between the two types of rapid reactions depends on the aurovertin concentration.

As with isolated F_1 [10], the rate constant of the slow phase in the fluorescence enhancement is independent of the aurovertin concentration.

Effects of ligands on the interaction between AS particles and aurovertin

Table I shows that pretreatment of AS particles with EDTA has little effect on the kinetics of the aurovertin fluorescence enhancement. $MgCl_2$ slightly quenches the final fluorescence level and enhances the rate of the slow phase (see also ref. 10). ADP slightly decreases the extent and rate of the fast phases, leaving the rate of the slow phase and the final fluorescence level almost unaffected. ATP strongly quenches the final fluorescence.

In the presence of $MgCl_2$, phosphate or ATP strongly quenches the final fluorescence, and the slow phase is abolished, while the rate of the fast phase is high, 70–80% of the total change being complete within 2–3 s. The slow phase completely disappears also with ADP.

It seems, therefore, that the extensive quenching of the aurovertin fluorescence is due to a quenching mainly of the slow phase and partly of the fast phase. This effect is maximal in the presence of $MgCl_2$ together with phosphate or ATP, indicating that a slow conformational change of F_1 is prevented by this combination of ligands.

TABLE I

EFFECT OF LIGANDS ON THE RATE AND EXTENT OF THE DIFFERENT PHASES OF AUROVERTIN FLUORESCENCE ENHANCEMENT IN AS PARTICLES

AS particles (0.34 mg protein/ml) were incubated in the standard medium for 1 min in the presence of the compounds listed, followed by the addition of 200 nM aurovertin. When ATP was present aurovertin was added immediately after the ATP. The fluorescence trace was analysed as described in Figs 1–3.

Additions	F_∞ (arbitrary units)	Initial jump (% F_∞)	Fast phase*		Slow phase $k \times 10^{-2}$ (s^{-1})
			% F_∞	$k \times 10^{-2}$ (s^{-1})	
None	95	27	78	3.0	0.8
$MgCl_2$ (1 mM)	80	30	72	3.3	1.6
$MgCl_2$, phosphate (5 mM)	31	70	100	7.0	0.0
$MgCl_2$, ADP (1 mM)**	68	50	100	4.5	0.0
$MgCl_2$, ATP (1 mM)	35	80	100	11	0.0
ADP (1 mM)	93	23	65	1.9	1.0
ATP (1 mM)	48	60	85	6.5	3.0
EDTA (2 mM)	98	26	70	2.1	0.7

* The percentage of the fast phase includes the initial jump.

** 5 units hexokinase were also present.

Effects of ligands on the fluorescence of the complex between A particles and aurovertin

Trace A of Fig. 4 shows the fluorescence enhancement when aurovertin is added to A particles suspended in the standard medium. When the fluorescence has reached a plateau, the addition of ADP gives rise to a rather complex transient change. In the presence of EDTA, which by itself does not alter the fluorescence level, ADP induces only a rapid enhancement (Trace F). Similar phenomena are observed with soluble F_1 (Van de Stadt, R. J. and Yeates, R. A., unpublished observations, see also ref. 9).

The addition of ATP causes a very fast quenching of the fluorescence (Traces B). The first 50–60% of the change is completed within 2 s. The concentration of ATP needed to give a half maximal quenching is very low, less than 1 μM (see ref. 10).

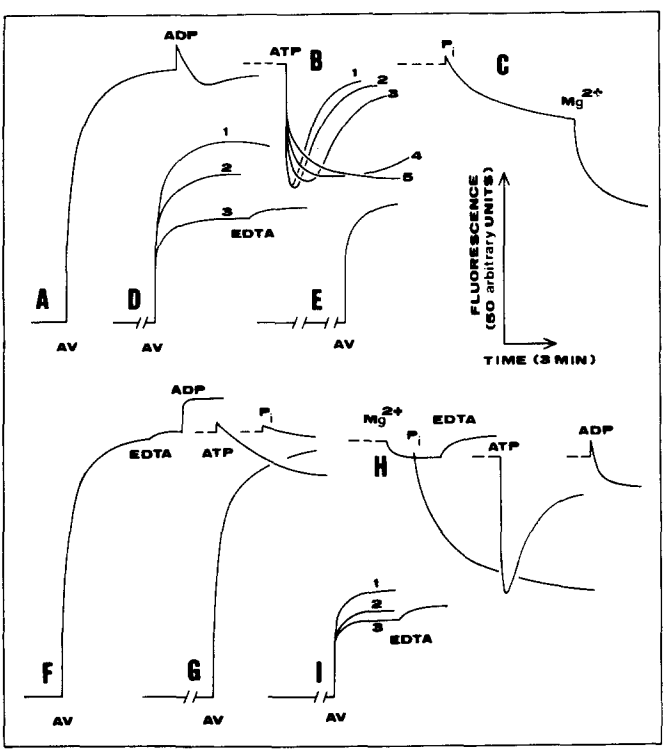

Fig. 4. Effect of ligands of the ATPase on the fluorescence of the complex between A particles and aurovertin. A particles (0.7 mg protein/ml) were incubated in the standard medium (see Methods and Materials). At the intersections marked AV, 200 nM aurovertin was added. The interruption of the traces before the addition of aurovertin refers to preincubation. Unless otherwise stated, the concentrations of the added compounds were: 1 mM $MgCl_2$, 1 mM ADP, 1 mM ATP, 5 mM potassium phosphate buffer (pH 7.3), 2 mM EDTA, 5 μg oligomycin. Further details: Trace B: concentrations of ATP (mM): Curve 1, 0.015; Curve 2, 0.05; Curve 3, 0.17; Curve 4, 0.50; Curve 5, 1.50. Trace D: preincubation times with phosphate were: Curve 1, 10 s; Curve 2, 2 min; Curve 3, 10 min. Trace E: after a preincubation for 10 min with phosphate, EDTA was added, followed by another incubation of 3 min. Trace G: after a preincubation for 3 min with EDTA, phosphate was added followed by another incubation of 10 min. Trace I: preincubated with $MgCl_2$ and phosphate for: Curve 1, 5 s; Curve 2, 40 s; Curve 3, 3 min.

With a low concentration of ATP this change is transient, due to the conversion of ATP into ADP, which hardly quenches (Trace A). At higher concentrations of ATP the fluorescence remains quenched. The quenching proceeds biphasically, the slower phase presumably being caused by the onset of a partial ATP hydrolysis. In the presence of EDTA the quenching of the fluorescence by 1 mM ATP is considerably less and much slower (Trace F). It proceeds in one phase with a first-order rate constant of 0.01 s^{-1}.

The mitochondrial ATPase, isolated in the presence of EDTA in a magnesium-free medium, contains 2 moles of tightly bound magnesium per mole of enzyme [23]. 'A' particles prepared in the presence of EDTA probably contain the same amount of tightly-bound magnesium per mole of F_1. The experiments reported indicate that this magnesium can be blocked by EDTA. The effect of EDTA is reversed by sedimenting the particles and removing the EDTA by washing. Thus, the transient effects of ADP and ATP seem to be dependent on the availability of enzyme-bound Mg^{2+}.

Oligomycin, added prior to the addition of ADP or ATP, has effects similar to those of EDTA (not shown in the figure). The quenching of the fluorescence by a high concentration of ATP was also very slowly overcome by oligomycin (one phase, $t_{\frac{1}{2}}$ 3 min) as has also been reported by Bertina [6].

In the presence of 1 mM $MgCl_2$, 1 mM ATP quenches the fluorescence very rapidly and extensively (Trace H). The fluorescence is rapidly restored as a result of hydrolysis of the ATP. In this trace it is also shown that $MgCl_2$ slightly quenches the fluorescence (one phase, $k = 0.032\ s^{-1}$), an effect that is reversed by EDTA (one phase, $k = 0.014\ s^{-1}$). Trace H also shows that in the presence of 1 mM $MgCl_2$, ADP induces a small initial rise in the fluorescence. This is followed by a more extensive quenching of the fluorescence than found in the absence of $MgCl_2$ (Trace A). Addition of AMP after or before the addition of aurovertin had little effect under any of the conditions tested.

The effect of phosphate on the aurovertin fluorescence is complex. 5 mM phosphate added to the complex of A particles and aurovertin quenches the fluorescence very slowly (Trace C, two phases, rate constants: 0.007 and 0.003 s^{-1}, respectively). The quenching is strongly enhanced by $MgCl_2$ (two phases; rate constants: 0.03 and 0.017 s^{-1}, respectively). In the presence of EDTA, phosphate has little effect (Trace F), indicating that magnesium is required. Traces D show that preincubation of the particles with phosphate for about 2 min leads to quenching of the fluorescence of subsequently added aurovertin by 50% of the maximal effect, whereas phosphate added after the aurovertin (Trace C) quenches the fluorescence by only 15% after 2 min. The quenching by phosphate is not reversed by EDTA (Traces D_3 and E), but it is prevented by EDTA (Trace G). Lardy and Lin [7] also observed a lower fluorescence of the soluble F_1-aurovertin complex in phosphate buffer as compared with Tris buffer (see also refs 10, 26).

The quenching due to preincubation with phosphate is enhanced and accelerated by the presence of $MgCl_2$ (Trace I). After 1 min 95% of the maximal effect is obtained. When phosphate is added to the complex of A particles and aurovertin in the presence of $MgCl_2$ (Traces H), the quenching of the fluorescence is less rapid: after 1 min only 40% of the maximal effect is obtained. The results indicate that the quenching of the aurovertin fluoresence by phosphate (1) requires magnesium, (2) is irreversible, unless ADP is added (see Fig. 5), (3) is slowed down by bound auro-

vertin. Furthermore, the quenching by phosphate is neither prevented nor overcome by oligomycin (not shown).

It is important to note that all the effects described for phosphate are observed only with phosphate or arsenate and are therefore not due to trivial changes in ionic strength. None of the quenching phenomena was induced by 20 mM Tris–acetic acid buffer, pH 7.3 (using 25 mM Tris–TES, pH 7.3, as the buffer in the standard medium) or by 20 mM of the following salts; KCl, NH_4Cl, KNO_3 or KCNS, or by 10 mM $KHCO_3$ or K_2SO_4. Because of the relatively high ionic strength of the standard medium (25 mM Tris–acetic acid buffer, pH 7.3) no appreciable effect was expected by increasing the ionic strength by 20 mM of monovalent salts. Bertina [6], however, found that when the complex of A particles and aurovertin was suspended in 3.3 mM glycylglycine buffer (pH 7.4), 50 mM KCl caused a decrease in the fluorescence that was fully accounted for by an increase in the dissociation constant of the complex and suggested that some charged species is involved in the aurovertin–protein interaction.

Competitive effects of phosphate, ADP and ATP on the fluorescence of aurovertin bound to A particles

In Traces A and B of Fig. 5, A particles were treated with 10 and 1 mM phosphate, respectively, prior to the addition of aurovertin. The resulting fluorescence is quenched to the same level as has been shown in Fig. 4 (the concentration of phosphate required for a half maximal effect is about 0.13 mM according to ref. 10). The rate and the extent of the fluorescence enhancement are dependent on the concentrations of both phosphate and ADP. Traces D and E represent a similar set of experiments in the presence of 1 mM $MgCl_2$ and 5 units of hexokinase. The rate of the fluorescence enhancement induced by ADP is much faster, and the extent still depends on the phosphate and ADP concentrations.

Trace C shows that oligomycin prevents the slow part of the fluorescence rise induced by ADP, but allows a fast and small enhancement similar to that shown in Fig. 4, Trace F. Finally, Trace B in Fig. 5 shows that when the ADP-induced change has occurred, oligomycin is able to reverse it slowly and partially.

These results suggest that ADP is able to replace phosphate on F_1 in a competitive manner. This replacement requires magnesium and is sensitive to oligomycin.

In Traces G, H and I of Fig. 5 different concentrations of ADP were added to the complex of A particles and aurovertin, followed by the addition of different concentrations of ATP. The rate and the extent of the fluorescence changes depend on the relative concentrations of ADP and ATP. Also in this case the quenching of the fluorescence by ATP is prevented by a prior addition of oligomycin. This competition can also be shown in the presence of EDTA (see also ref. 9). Finally, Trace F shows the reverse experiment. When the aurovertin fluorescence is quenched by $MgCl_2$ plus phosphate, the addition of 1 mM ATP gives only a small enhancement (also reported by Lardy and Lin [7]) and this is followed by a breakdown of ATP into ADP plus phosphate with subsequent replacement of ATP on F_1 by ADP. As expected, this change is sensitive to oligomycin.

Binding of aurovertin to A particles

Titrations of A particles with aurovertin were performed in separate experi-

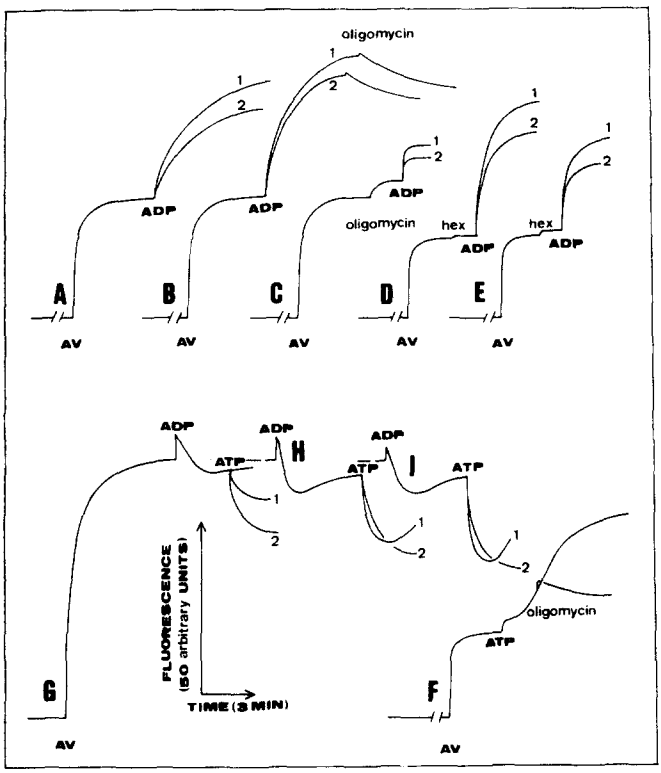

Fig. 5. Competitive effects of phosphate, ATP and ADP on the fluorescence of the complex between A particles and aurovertin. The same conditions as under Fig. 4. Further details: Trace A: 10-min preincubation with 10 mM phosphate: Curve 1, 1.5 mM ADP; Curve 2, 0.15 mM ADP. Traces B and C: the same as Curve A, but with 1 mM phosphate. Trace D: 3-min preincubation with 1 mM phosphate in presence of $MgCl_2$, 5 units hexokinase were added where shown; Curve 1, 1.5 mM ADP; Curve 2, 0.15 mM ADP. Trace E: the same as Curve D, but with 10 mM phosphate. Trace F: 3-min preincubation with 1 mM phosphate in the presence of $MgCl_2$. Trace G: 1.5 mM ADP; Curve 1, 0.15 mM ATP; Curve 2, 1.5 mM ATP. Trace H: 0.15 mM ADP, otherwise as Trace G. Trace I: 0.05 mM ADP, otherwise as Trace G.

ments at varying concentrations of protein. Figs 6A and 6B represent double-reciprocal plots of the observed final fluorescence versus the protein concentration at different concentrations of aurovertin, in the absence and presence of 5 mM phosphate, respectively. Extrapolation to infinite protein concentration gives the fluorescence yield expected when all aurovertin is bound to the A particles [22]. If the fluorescence yield at infinite protein concentration is plotted versus aurovertin concentration added (Fig. 6C) a straight line is obtained, allowing the calculation of the relative quantum yield (Q_{rel}), expressed in arbitrary units/nM aurovertin.

The values for Q_{rel} can be used for the calculation of a Scatchard plot [23] from a titration with aurovertin at a fixed concentration of protein (Fig. 6D). Values obtained for the dissociation constants of the aurovertin–A particle complex (K_D) and concentration of binding sites for aurovertin (n) are listed in Table II.

In order to test the validity of this experimental method for the determination

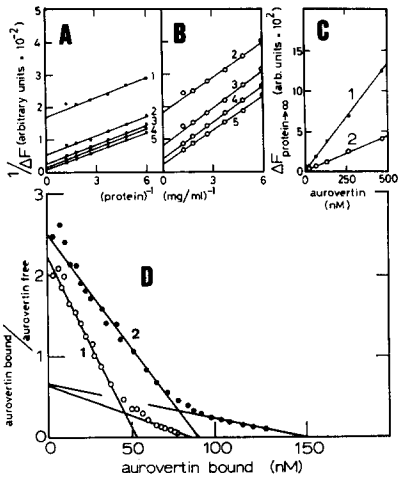

Fig. 6. Binding of aurovertin to A particles as measured by the fluorescence of bound aurovertin. A. Standard medium. Double-reciprocal plot of the final fluorescence level versus the protein concentration. Concentrations of aurovertin (nM): 1, 20; 2, 67; 3, 133; 4, 267; 5, 467. B. 5 mM potassium phosphate buffer (pH 7.3) was included in the standard medium. Other conditions are the same as in A. C. Plot of the extrapolated fluorescence yield at infinite protein concentration versus the aurovertin concentration. This graph yields the relative quantum yield Q_{rel} (units/nM aurovertin); 1, standard medium; 2, plus phosphate. D. Scatchard plot of the binding of aurovertin to A particles. Titration with aurovertin (23 concentrations ranging from 5 to 1400 nM) was done with 0.37 mg protein/ml. Each concentration of aurovertin was a separate experiment. Relative quantum yields, obtained under C, were used for the calculations.

of binding data from the fluorescence yield of bound aurovertin, the binding was also determined by measuring the concentration of aurovertin free in solution after spinning down the particles (Fig. 7). This could be done very sensitively by comparing the fluorescence in the presence of an excess of A particles with that of known concentrations of aurovertin (see legend Fig. 7). The binding data obtained in this way in the absence and presence of phosphate are also listed in Table II.

TABLE II

PARAMETERS OF BINDING OF AUROVERTIN TO A PARTICLES

n_1 stands for the concentration of strong aurovertin-binding sites, n_2 for the concentration of weaker binding sites, i.e. total sites minus strong sites, K_{D_1} and K_{D_2} are dissociation constants for these sites and Q_{rel} is the relative quantum yield.

Calculated from	Additions	Q_{rel} (arbitrary units/nM aurovertin)	n (nmole/mg of protein)		K_{D_1} (nM)	K_{D_2} (nM)
			n_1	n_2		
Fig. 6	None	2.6	0.15	0.10	24	135
	Phosphate	0.9	0.25	0.17	37	240
Fig. 7	None	–	0.16	0.14	14	115
	Phosphate	–	0.27	0.16	17	270

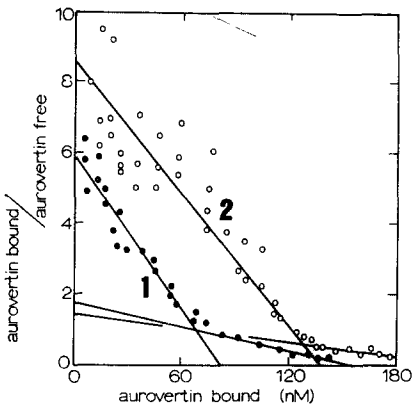

Fig. 7. Binding of aurovertin to A particles as measured by the concentration of free aurovertin. In a final volume of 4 ml, A particles (0.51 mg protein /ml) were incubated at 30 °C in a standard medium (Curve 1) or in a standard medium supplemented with 5 mM potassium phosphate buffer (pH 7.3) (Curve 2). After the addition of aurovertin (18 different concentrations ranging from 10 to 1800 nM) incubation was continued till the fluorescence was constant (usually after 10 min). Samples were then centrifuged at 30 °C for 15 min at 200 000 × *g*. Supernatants were kept on ice. The aurovertin content of the samples was measured indirectly. A calibration curve was made by adding A particles (0.51 mg protein/ml), either untreated or preincubated for 15 min with 5 mM phosphate, to standard medium supplemented with aurovertin concentrations ranging from 2 to 100 nM, and the final fluorescence was plotted versus aurovertin concentration. The same concentration of A particles was then added to appropriate dilutions of the supernatants (at least in duplicate) and the fluorescence measured. The calculated concentration of free aurovertin in the supernatants allowed the calculation of the Scatchard plot. Corrections were made for binding of aurovertin to the centrifuge tubes (binding, which was proportional to the aurovertin concentrations, was less than 15 %). In the presence of phosphate the points are much more scattered. This is due to the fact that phosphate quenches the aurovertin fluorescence (see Fig. 4), making the experimental errors larger.

The two methods gave comparable results and indicated the presence of two binding sites for aurovertin on the particles, one with a high affinity (n_1) and one with a low affinity (n_2).

Phosphate strongly quenches the fluorescence (Fig. 4). As shown in Table II this is caused mainly by a decrease by 65 % of the relative quantum yield. Furthermore, the concentration of both strong and weak binding sites increases, and the affinity of both sites for aurovertin decreases. Although the fluorescence extrapolation method (Fig. 6) gives more accurate binding data, the increase of the concentration of binding sites induced by phosphate is also clearly indicated by the direct method (Fig. 7). This rules out the possibility that in the fluorescence extrapolation method the increase in the concentration of sites is merely the result of the decreased relative quantum yield introduced in the calculations. Moreover, Fig. 6C shows that the relative quantum yield is the same whether 20 nM or 467 nM aurovertin is used, suggesting that this parameter is equal for both the strong and weak binding sites (the calculation of the Scatchard plot in Fig. 6D is based upon this assumption).

Several authors [6–10] have found that aurovertin forms a stoicheiometric complex (1 : 1) with soluble F_1 under standard conditions. None of the calculated Scatchard plots for the binding of aurovertin to F_1 is curved [8, 9]. Recently published dissociation constants are 40 [9] and 59 nM [8]. On the other hand, Bertina [6] found

a 50% increase in the concentration of binding sites of aurovertin to A particles upon addition of phosphate.

For the experiments listed in Table III, a single batch of AS particles, stored under liquid nitrogen in small aliquots, was used. In control experiments it was shown that this storage had no effect on the binding properties.

In Table III the effects of ligands of F_1 on the binding parameters of aurovertin to AS particles (measured by the method described in Fig. 6) are listed. Under standard conditions (see Methods and Materials) aurovertin binds to AS particles with a high relative quantum yield, and most of the fluorochrome binds to strong binding sites. $MgCl_2$ quenches the fluorescence slightly (cf. Fig. 4) and has only a small effect on the binding parameters. As shown before, phosphate, particularly in the presence of Mg^{2+}, brings the ATPase in a conformation that binds aurovertin with a decreased relative quantum yield and decreased affinity, while the concentration of binding sites, both strong and weak, is doubled.

ATP alone or in the presence of $MgCl_2$, with or without phosphate, produces the same kind of changes as described for $MgCl_2$ plus phosphate. In the presence of 2 mM EDTA also, ATP diminishes the relative quantum yield, but only by 30% (cf. Fig. 4). Bertina [6] also reported that ATP increases the concentration of binding sites (50%) in A particles, while it decreases the affinity and relative quantum yield. Interestingly, Chang and Penefsky [9] reported recently that ATP is able to induce a second binding site for aurovertin also in soluble F_1, both sites having the same strongly decreased affinity (K_D increases from 40 to 520 nM; the relative quantum yield decreased by 35%). It is also possible to find an increase in the number of bind-

TABLE III

EFFECT OF LIGANDS ON THE AUROVERTIN BINDING PARAMETERS TO AS PARTICLES

All other additions were made prior to the addition of aurovertin, except for ATP which was added after the fluorescence had achieved a final level (in the presence of $MgCl_2$ plus or minus phosphate). All calculations were made on final fluorescence levels (i.e. at least pseudo-equilibrium). Binding data were obtained as described under Fig. 6. Titrations with aurovertin were carried out with at least 18 different concentrations (5–1200 nM) in separate experiments with 0.34 mg AS particles per ml. Symbols used in the table are explained in Table II.

Additions	Q_{rel} (arbitrary units/nM aurovertin)	n (nmole/mg of protein)		K_{D_1} (nM)	K_{D_2} (nM)
		n_1	n_2		
None	2.3	0.20	0.06	26	100
$MgCl_2$ (1 mM)	2.1	0.19	0.06	35	125
Phosphate (5 mM)	0.9	0.35	0.15	50	185
$MgCl_2$, phosphate	0.7	0.30	0.20	80	340
ADP (1 mM)	1.7	0.28	0.07	35	115
ATP (1 mM)	0.9	0.35	0.21	95	320
$MgCl_2$, ADP*	1.4	0.25	0.20	25	140
$MgCl_2$, ADP, phosphate*	1.2	0.28	0.17	55	210
$MgCl_2$, ATP	0.7	0.39	0.26	85	290
$MgCl_2$, ATP, phosphate	0.7	0.37	0.24	98	330

* Hexokinase (5 units) also added.

ing sites and in the dissociation constant for the aurovertin complex with soluble F_1 upon addition of phosphate (Yeates, R. A., personal communication).

ADP also reduces the relative quantum yield somewhat. In the presence of EDTA, ADP leaves the relative quantum yield the same or slightly enhances it, as compared to the control experiment (not shown). A similar observation was described for isolated F_1 [9]. In the presence of $MgCl_2$, ADP diminishes the relative quantum yield but not as strongly as phosphate or ATP, and has hardly any effect on the affinity. The same is reported for isolated F_1 [9]. ADP increases the concentration of binding sites, predominantly the strong sites, but the concentration of total sites remains smaller than that induced by phosphate or ATP. In the presence of $MgCl_2$ and ADP, more relatively weak binding sites are exposed, but the affinities are little affected. When phosphate is present as well, competitive interactions can be seen between phosphate and ADP (cf. Fig. 5).

Qualitatively, the same results were obtained with A particles (see Methods and Materials) as with AS particles.

DISCUSSION

An increase of the fluorescence of the antibiotic aurovertin has been observed when it is added to rat-liver and rat-heart mitochondria [6, 8], submitochondrial particles and F_1 derived from beef-heart [6–10] or yeast mitochondria (Out, T. A. and Bertina, R. M., unpublished observations). It is not reactive with chloroplasts or isolated chloroplast CF_1 or with the ATPase isolated from *Escherichia coli* (Kraayenhof, R., Nieuwenhuis, F. J. R. M. and Van de Stadt, R. J., unpublished observations). It also does not react with bovine serum albumin or lecithin [6] or mitochondrial components other than F_1 [7]. This suggests that for interaction a rather specific micro-environment is required on the enzyme, and also that the conformation of the active centre of the enzyme in chloroplasts and bacteria is rather different from that in mitochondria. Judging by the sensitivity of the fluorescence of the bound aurovertin to substrates (ADP, ATP, P_i) of the enzyme and to the co-factor Mg^{2+}, it seems likely that it binds near the active centre of the enzyme, or that the aurovertin-binding site responds to changes at the active centre.

Effect of ligands on the fluorescence of the particle–aurovertin complex

The fluorescence enhancement accompanying binding of aurovertin to AS particles is triphasic, a very rapid first phase, completed within 2–3 s, preceding the fast second and slow third phases (Fig. 1). The extent of the first phase and the rate of the second phase are dependent on the aurovertin concentration. Two possibilities may be considered. (1) The first and second phases represent binding of aurovertin to the two sites revealed by binding measurements. (2) The first phase represents rapid binding to one site, and the second phase is the result of a redistribution on to a second binding site, induced by aurovertin.

The rate of the slow third phase is not dependent on the aurovertin concentration. Ligands like ATP and phosphate prevent the occurrence of this phase (Table I) and simultaneously diminish the relative quantum yield of bound aurovertin and increase the binding capacity of the particles (Table III). The slow phase in the aurovertin fluorescence change may be the result of a slow conformational change of

F_1 induced by aurovertin, as suggested by Yeates [10].

In accordance with Chang and Penefsky [9], we shall interpret changes in aurovertin fluorescence as the result of conformational changes in F_1, induced by ligands of the enzyme only.

Based upon the competing effects of ligands on the aurovertin fluorescence (Fig. 5), it is likely that there is at least one binding site on F_1, where ATP, ADP or phosphate can bind competitively (cf. ref. 9). This binding site might be identical to the active region of the enzyme. Binding of ADP has relatively small effects on the fluorescence of the particle–aurovertin complex, whereas ATP and phosphate quench it strongly.

The results shown in Figs 4 and 5 suggest that replacement of one ligand by another is dependent on the availability of accessible enzyme-bound or free Mg^{2+}. EDTA or other Mg^{2+}-chelating compounds, like ATP in sufficient concentration, may reversibly block the tightly bound magnesium. In the presence of EDTA, ADP induces only a rapid enhancement of the fluorescence; ATP induces a slow and small quenching, and the quenching by phosphate is prevented. This suggests that Mg^{2+} is involved in the effect of these ligands on the conformation of F_1. Adolfsen and Moundrianakis [26] reported recently that Mg^{2+} activation of the ATPase activity of the heat-labile coupling factor from *Alcaligenes faecalis*, involves formation of a readily reversible metal–enzyme complex. Inhibition of the activation by EDTA is due to reversible complex formation with the enzyme–metal complex and not to removal of bound metal from the enzyme.

The quenching of the aurovertin fluorescence by phosphate is induced slowly, the rate being strongly stimulated in the presence of Mg^{2+}. Interestingly, the fluorescence change induced by phosphate occurs more rapidly if the particles are preincubated with phosphate (Fig. 4). This might be correlated with the finding of Mitchell and Moyle [27] that aurovertin prevents the phosphate-induced stimulation of the ATPase activity in submitochondrial particles.

Judging by its effects on the phosphate-induced quenching, oligomycin, an energy-transfer inhibitor that binds to the membrane part of the ATPase complex [28] does not prevent phosphate occupying the binding site, but it prevents ADP from replacing phosphate (Fig. 5).

It also prevents and reverses the ATP-induced quenching of the fluorescence (not shown). This suggests that oligomycin favours a conformation of the enzyme that can only bind ADP (and phosphate) but not ATP. Because oligomycin and ligands of F_1 bind to different sites, this action of the antibiotic must proceed through a conformational change of F_1 imposed by an oligomycin-induced change in the membranous part of the ATPase complex (see ref. 6).

The effect of ligands on the binding of aurovertin to submitochondrial particles

In all the experiments reported in this paper, we have found two binding sites for aurovertin in submitochondrial particles, one with a high affinity (n_1) and one with a low affinity (n_2). Chang and Penefsky [9] have found two aurovertin-binding sites per molecule on isolated F_1 in the presence of ATP and EDTA. In a medium containing EDTA plus ADP, or $MgCl_2$, one aurovertin-binding site on F_1 was found. One binding site on F_1 has been reported by several authors [6–9].

Other information reveals that also phosphate (in the absence of EDTA) is

able to increase the concentration of aurovertin-binding sites in A particles [6] or isolated F_1 (Yeates, R. A., personal communication). It is well known that inhibition of the ATPase activity of submitochondrial particles requires about 10 times as much aurovertin as required for the inhibition of oxidative phosphorylation [2–6]. Chang and Penefsky [9] suggested that the second aurovertin-binding site induced by ATP might be the site involved in the inhibition of ATPase activity, whereas the other site is involved in the inhibition of oxidative phosphorylation. A model to explain the results of the binding experiments listed in Table III is presented in Fig. 8. This model applies to endogenously uncoupled A or AS particles, which cannot carry out ATP synthesis.

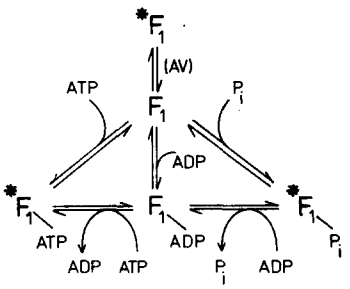

Fig. 8. Scheme of the interaction between ligands and the ATPase in endogenously uncoupled AS particles. AV stands for aurovertin.

Two conformations of F_1 are in equilibrium, termed F_1 and $*F_1$. The F_1 conformation contains only aurovertin-binding sites of the strong type (n_1, one site per mole of enzyme), that may be involved in the inhibition of oxidative phosphorylation. The affinity of this site for aurovertin is high and the conformation of the enzyme permits the fluorochrome to bind with a high quantum yield. The second conformation of the enzyme ($*F_1$) exposes two binding sites for aurovertin, one of the first type (n_1) and one of the second type (n_2) per mole of enzyme. The second binding site may be involved in the inhibition of the ATPase activity. The affinity of the stronger binding site in this conformation is less than that of the strong binding site in the F_1 conformation. Aurovertin binds to the $*F_1$ conformation with a low quantum yield.

Changes of the fluorescent properties of the aurovertin–particle complex can be explained qualitatively by a shift in the equilibrium between the two conformations of F_1, induced by binding of ligands of the enzyme.

The binding data listed in Table III may now be explained in the following way: ATP and phosphate, especially in the presence of Mg^{2+}, induce conformation $*F_1$. ADP, in particular if it is bound in the presence of EDTA, stabilizes conformation F_1. In the absence of EDTA, endogenous magnesium interferes, inducing a partial shift to the $*F_1$ conformation. This effect is more pronounced with added Mg^{2+}, although the affinities and the quantum yield remain relatively high. When phosphate is also introduced, competition between ADP and phosphate becomes apparent and the equilibrium is shifted towards conformation $*F_1$.

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