Biochimica et Biophysica Acta, 347 (1974) 253–263

BBA 46735

BINDING OF AUROVERTIN TO PHOSPHORYLATING SUBMITOCHONDRIAL PARTICLES

R. J. VAN DE STADT and K. VAN DAM

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)

(Received November 14th, 1973)

SUMMARY

1. The slow enhancement of the fluorescence of the complex between aurovertin and Mg–ATP particles, induced by succinate oxidation in the absence of adenine nucleotides and phosphate, is completely accounted for by dissociation of the natural ATPase inhibitor from the particles.

2. The large increase in the aurovertin fluorescence induced by an anaerobic–aerobic transition requires the presence of all State-3 components (substrate, ADP, phosphate and Mg^{2+}). This change is very rapid with a half-time of 20 ms.

3. There are two aurovertin-binding sites on Mg–ATP particles, one with high affinity and one with low affinity. Binding of aurovertin to the strong sites is positively cooperative only under State-3 conditions.

4. The quenching of the fluorescence of the particle–aurovertin complex induced by an uncoupler is rapid and extensive in the presence of ATP. The rapid quenching induced by an uncoupler is followed by a slow increase in the aurovertin fluorescence, that may be attributed to a redistribution of bound adenine nucleotides. All uncoupler-induced changes are prevented by oligomycin.

5. Excess oligomycin added to Mg–ATP particles oxidizing succinate under State-3 conditions initially induces a rapid enhancement of the aurovertin fluorescence. The pH dependency of this change suggests that this rise may be attributed to the induction of a maximally 'coupled' membrane.

INTRODUCTION

Phosphorylating Mg–ATP particles [1] contain a relatively large amount of a small protein, the ATPase inhibitor [2], that binds specifically to F_1 [2, 3]. Van de Stadt and co-workers [4, 5] showed the existence of a reversible equilibrium between

Abbreviations and symbols: F_1, mitochondrial ATPase; Mg–ATP particles, phosphorylating submitochondrial particles prepared by sonication of beef-heart mitochondria in the presence of 15 mM $MgCl_2$ and 1 mM ATP (pH 7.5); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 1799, α,α′-bis(hexafluoroacetonyl)acetone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

the mitochondrial ATPase (F_1) and its inhibitor. Lardy and Lin [6] have shown that the antibiotic aurovertin, a potent inhibitor of oxidative phosphorylation [7], also binds specifically to F_1. Binding is accompanied by at least a 50-fold enhancement of the fluorescence yield of the complex. The fluorescence properties of aurovertin bound to F_1 are strongly influenced by ligands of the ATPase enzyme [8–12]. Chang and Penefsky [9] and Bertina et al. [10] reported that the fluorescence yield of aurovertin bound to submitochondrial particles or intact mitochondria is increased upon energization of the membrane. The latter authors showed that aurovertin binds cooperatively to State-3 mitochondria. It has been generally agreed [8–12] that changes of the fluorescence of aurovertin monitor conformational changes of the ATPase, induced by ligands of the enzyme or by the membrane itself.

The aim of this paper is to examine the nature of the changes in the fluorescence yield of aurovertin, bound to Mg–ATP particles, that take place upon energization and de-energization of the mitochondrial membrane.

MATERIALS AND METHODS

Preparations

Heavy beef heart-mitochondria [4] and Mg–ATP particles [1] were prepared according to the published procedures. Aurovertin D was isolated in this laboratory in pure form as described by Bertina [8]. Physical data have been reported earlier [8, 11, 12].

Fluorometric measurements

The enhancement of aurovertin fluorescence accompanying binding of the antibiotic to Mg–ATP particles was recorded in a specially adapted Eppendorf fluorimeter, using the same filters as described earlier [12]. The standard medium contained, in a final volume of 1.5 ml, 375 μmoles sucrose, 37.5 μmoles glucose and 37.5 μmoles Tris–acetic acid buffer (pH 7.3). Reactions were carried out at 30 °C. The fluorimeter was adjusted to the same sensitivity as used for experiments published before [5, 12], by calibration with a standard solution of NADH.

Kinetics of fluorescence changes

Kinetics of the aurovertin fluorescence changes were recorded in a fluorimeter made up of the optical assembly of a Perkin-Elmer MPF-2A spectrofluorimeter, fitted with a closed and thermostatted cylindrical quartz cuvette (front-face geometry), that can be rapidly stirred by an overhead Teflon stirrer. Additions were made by microlitre syringes directly into the vortex, the complete mixing time being less than 50 ms. The signals from both sample and reference photomultipliers were appropriately amplified and filtered using Analog 40 J operational amplifiers (response time 100, 10 or 1 ms) and read-out by a Tektronix R 5103 N storage oscilloscope. Excitation, 370 nm (band width, 6 nm); emission, 470 nm (band width, 15 nm).

Analytical methods were the same as described previously [4].

Materials

ATP, ADP, hexokinase and catalase were obtained from Boehringer und Söhne. Oligomycin was gift of by Upjohn Chemical Co., the uncouplers α,α′-bis

(hexafluoroacetonyl)acetone (1799) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were gifts from Dr P. Heytler. 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was a gift from Dr R. H. Büchel. All other chemicals were of analytical grade. Hexokinase was dialysed before use as described before [5]. All organic acids used were neutralized with Tris to pH 7.3.

RESULTS

The effect of State-3 components on the fluorescence of aurovertin bound to Mg–ATP particles

In Fig. 1, Trace B, the fluorescence is recorded upon addition of aurovertin to Mg–ATP particles, suspended in the standard medium. The addition of succinate initiates a slow rise in the fluorescence. The rate of this enhancement is slowed by malonate (rate of succinate oxidation decreased by 80%). Moreover, anaerobiosis did not reverse the change while KCN reversed it only to a small extent. Traces A of Fig. 1 demonstrate that the extent of the fluorescence rise reached soon after the addition of aurovertin increases greatly with the time of preincubation of the particles with succinate. Trace C shows that KCN prevented this effect. The uncoupler 1799 and the inhibitor oligomycin also prevent the succinate-induced fluorescence rise (Trace D), but, as shown in Trace B, had a relatively small effect when added after the succinate-induced fluorescence increase was complete.

When phosphate is added to the complex between Mg–ATP particles and aurovertin, incubated in the standard medium supplemented with succinate and $MgCl_2$, the aurovertin fluorescence is strongly quenched, and ADP partly reverses

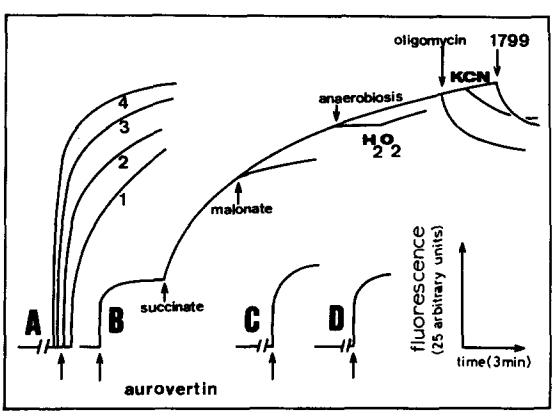

Fig. 1. Effect of succinate oxidation on the aurovertin fluorescence in the presence of Mg–ATP particles. Aurovertin (200 nM) was added to Mg–ATP particles (0.7 mg protein) suspended in 1.5 ml standard medium (see Materials and Methods), supplemented with 1 mM $MgCl_2$ and 50 units catalase. Trace A: particles were preincubated with 10 mM succinate (pH 7.3), before the addition of aurovertin. Preincubation times: (1) 30 s; (2) 3 min; (3) 8 min; (4) 15 min. Every 5 min 0.5 mM H_2O_2 was added. Trace B: 10 mM succinate, 2 mM malonate, 0.5 mM H_2O_2, 2 mM KCN, 13 μM 1799 and 5 μg oligomycin were added at the indicated points. Trace C: particles were preincubated for 8 min in presence of 2 mM KCN and 10 mM succinate before the addition of aurovertin. Trace D: particles were preincubated for 8 min with 13 μM 1799 or 5 μg oligomycin and 10 mM succinate, before the addition of aurovertin. 0.5 mM H_2O_2 was added every 4 min.

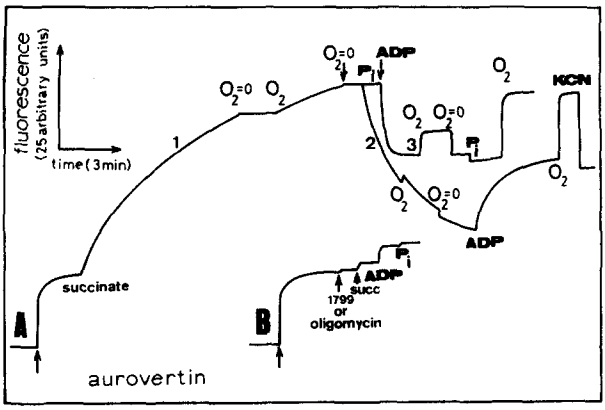

Fig. 2. Influence of State-3 components and anaerobiosis on the aurovertin fluorescence in the presence of Mg–ATP particles. Aurovertin (200 nM) was added to Mg–ATP particles (0.75 mg protein), suspended in 1.5 ml standard mixture (see Materials and Methods), supplemented with 1 mM $MgCl_2$ and 50 units catalase. Further additions at the points indicated in the figure: 10 mM succinate (pH 7.3), 5 mM phosphate buffer (pH 7.3), 1 mM ADP, 13 μM 1799, 5 μg oligomycin and 0.3 mM H_2O_2.

this change (Trace A2 of Fig. 2). Trace A3 shows that ADP quenches the fluorescence to a smaller extent than phosphate. The extent of the ADP-induced quenching could be considerably diminished by hexokinase, suggesting that the quenching is brought about by ATP formed from the ADP by adenylate kinase, known to be present in Mg–ATP particles. ATP and phosphate quench the aurovertin fluorescence, when bound to either submitochondrial particles or isolated F_1, while ADP slightly enhances it [9, 11, 12].

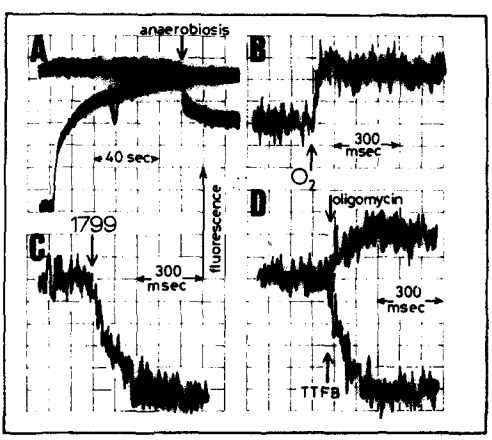

Fig. 3. Kinetics of the aurovertin fluorescence change in Mg–ATP particles. Mg–ATP particles (1.5 mg protein) were suspended in 1.1 ml buffer at 25 °C, containing 250 μmoles sucrose, 25 μmoles glucose, 25 μmoles Tris–acetic acid buffer, 2 μmoles $MgCl_2$, 5 μmoles phosphate buffer, 1 μmole ADP, 10 μmoles succinate, 10 units hexokinase and 1000 units catalase at a final pH of 7.3. After 2 min, 0.3 nmole aurovertin was added (A). The suspension was kept aerobic by appropriate additions of 0.5 μmole H_2O_2. Further additions as indicated in the figure: 1.0 μmole H_2O_2, 14 nmoles 1799, 2.5 nmoles TTFB and 8 μg oligomycin. In A, the electronic response time was set at 100 ms; in B, C and D at 10 ms. In C and D, oligomycin, 1799 or TTFB were added aerobically.

It can be seen in Traces A1, A2 and A3 of Fig. 2 that anaerobic–aerobic transitions gave large fluorescence changes only under State-3 conditions. The kinetics of the enhancement under State-3 conditions in an oxygen-pulse experiment are recorded in Fig. 3B. The half-time is about 20 ms. Trace B of Fig. 2 illustrates that 1799 or oligomycin added before succinate not only prevented the succinate-induced rise in the aurovertin fluorescence, but also minimized the effect of ADP (cf. Fig. 4).

Effect of an uncoupler and oligomycin on the aurovertin fluorescence in Mg–ATP particles

In Fig. 4, Trace A, aurovertin was added to Mg–ATP particles supplemented with Mg^{2+} and hexokinase. After the addition of phosphate, ADP induces a slow enhancement of the aurovertin fluorescence. When an uncoupler is added prior to ADP this slow rise is not observed (Fig. 2B). After the fluorescence had reached a final level, the addition of an uncoupler induced a small and rapid quenching of the fluorescence, followed by a slow increase (Fig. 4, Trace A1). When hexokinase was omitted (Trace B), the rapid quenching that precedes the slow rise was much larger.

Trace C shows that the addition of ATP instead of ADP also induces a slow rise in the aurovertin fluorescence. The addition of an uncoupler induces an immediate and nearly complete quenching of the increased fluorescence. A subsequent slow rise sets in after a lag period. (Under these conditions the ATPase activity is increased at least 5-fold [4]). The results suggest that the extent of the rapid quenching induced by an uncoupler is increased when more ATP is available.

Trace A2 (Fig. 4) illustrates that the high fluorescence level obtained under State-3 conditions can be reached independent of the order of additions (cf. Figs 2 and 5). Under these conditions an uncoupler induces a rapid and large quenching of the fluorescence. The kinetics of this change are recorded in Fig 3, Traces C and D. The half-times for the uncouplers 1799 and TTFB are about 50 and 40 ms, respec-

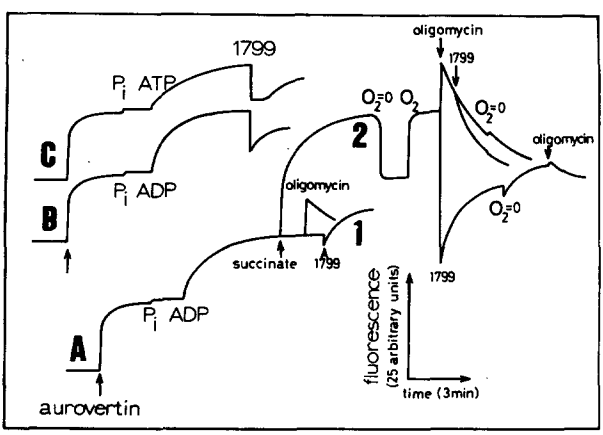

Fig. 4. Effect of an uncoupler and oligomycin on the fluorescence of aurovertin in the presence of Mg–ATP particles. The incubation conditions were the same as described under Fig. 2. Further additions at the points indicated in the figure: 10 mM succinate (pH 7.3), 5 mM phosphate buffer (pH 7.3), 1 mM ADP, 1 mM ATP, 13 μM 1799, 5 μg oligomycin and 0.3 mM H_2O_2. In Trace A the standard mixture was supplemented with 5 units hexokinase.

tively. The rapid quenching induced by an uncoupler is followed again by a slow and extensive rise in the fluorescence. Anaerobiosis allows the aurovertin fluorescence to increase even more, after a small transient quenching of the fluorescence. This complex change is also induced by other uncouplers such as by TTFB or FCCP.

As also shown in Trace A2 of Fig. 4, oligomycin induces a fast rise in the aurovertin fluorescence with a half-time of about 50 ms (Fig. 3D). This enhancement is followed by a slow quenching. It is of interest to note that in the presence of oligomycin neither anaerobiosis nor the addition of an uncoupler induce a rapid quenching of the fluorescence. The changes induced by an uncoupler or oligomycin are qualitatively comparable in the absence or presence of succinate (Fig. 4, Traces A1 and A2).

A low pH induces better coupling of the membrane in submitochondrial particles [13, 14]. Since it appeared possible that the effects of oligomycin and uncoupler on the aurovertin fluorescence in Mg–ATP particles suspended under State-3 conditions might depend on the degree of coupling of the membrane, they were recorded at different pH values (Fig. 5). It can be seen that if ADP is added to the complex of aurovertin and particles oxidizing succinate in the presence of $MgCl_2$ and phosphate, the rate and extent of the fluorescence enhancement is considerably larger at low pH. Furthermore, the extent of the anaerobic–aerobic transitions is larger at low pH.

It is also illustrated in Fig. 5 that at low pH the oligomycin-induced increase in the aurovertin fluorescence is relatively small, while the 1799-induced quenching is large. Both changes proceeded to about the same fluorescence levels over the whole pH range tested.

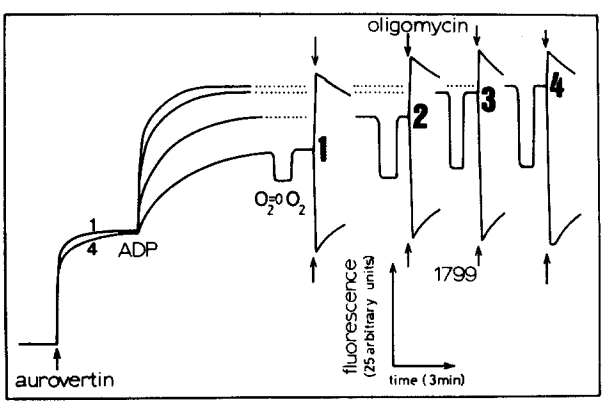

Fig. 5. pH dependence of the oligomycin- and 1799-induced change of the aurovertin fluorescence in the presence of Mg–ATP particles. Aurovertin (200 nM) was added to Mg–ATP particles (0.7 mg protein) suspended in 1.5 ml standard medium (see Materials and Methods) supplemented with 1 mM $MgCl_2$, 5 mM phosphate buffer, 10 mM succinate, 50 units catalase and 5 units hexokinase. Before the assay the complete mixture except for the enzymes was adjusted with 2 M acetic acid or 2 M Tris to the following pH values: Curve 1, 8.0; Curve 2, 7.5; Curve 3, 7.0; Curve 4, 6.5. Further additions at the points indicated in the figure: 1 mM ADP (at corresponding pH), 10 μM 1799 or 5 μg oligomycin and 0.3 mM H_2O_2.

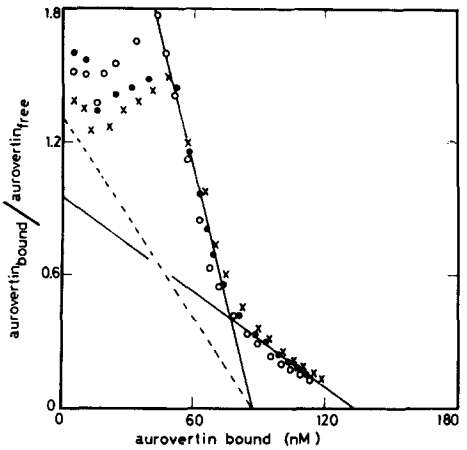

Fig. 6. Scatchard plot for the binding of aurovertin to phosphorylating Mg–ATP particles, oxidizing succinate. The conditions of the experiment and the order of additions are given in Table I. Mg–ATP particles (0.33–0.35 mg protein/ml) were titrated with aurovertin in separate experiments (18 different concentrations ranging from 5 to 1000 nM). The relative quantum yield (Q_{rel}) was calculated from a double-reciprocal plot of the final fluorescence versus the protein concentration (0.18–0.70 mg protein/ml) at 4 different aurovertin concentrations (40–400 nM), in essentially the same way as described before [5, 12]. From the relative quantum yield and the titration curve, the Scatchard plot [15] was calculated. The experiment was done in triplicate with different batches of freshly prepared particles. Calculated binding parameters are listed in Table I.

TABLE I

PARAMETERS OF BINDING OF AUROVERTIN TO Mg–ATP PARTICLES

All binding data were obtained by measurement of the final fluorescence of bound aurovertin by the extrapolation method described under Fig. 6. A standard fluorescence trace is illustrated in Fig. 4, Trace A2. (1) Aurovertin was added to Mg–ATP particles suspended in 1.5 ml standard medium supplemented with 1 mM $MgCl_2$, 50 units catalase and 7.5 units hexokinase. All subsequent additions were made after the fluorescence had reached a constant value. (2) 5 mM phosphate buffer (pH 7.3). (3) 1 mM ADP. (4) 5 mM succinate (pH 7.3); when required 0.3 mM H_2O_2 was added to keep the suspension aerobic. (5) The suspension reached anaerobiosis. (6) 30 s after the addition of 0.3 mM H_2O_2, 13 μM 1799 was added. Q_{rel} is the relative quantum yield; n_1, concentration of strong aurovertin-binding sites; n_2, concentration of weak aurovertin-binding sites; K_{D_1}, dissociation constant of the strong binding site (cooperativity is indicated by a decrease in dissociation constant); K_{D_2}, dissociation constant of the weak binding site.

Expt	Consecutive additions or events	Q_{rel} (units/nM aurovertin)	n_1 (nmole/mg protein)	n_2	K_{D_1} (nM)	K_{D_2} (nM)
1	None	0.5	0.18	0.40	42	390
2	Phosphate	0.5	0.14	0.41	25	370
3	ADP	0.75	0.14	0.50	11	330
4	Succinate	1.6	0.25	0.12	65 → 20	140
5	$O_2 = 0$	0.95	0.13	0.42	10	175
6	1799, $O_2 = 0$	1.1	0.27	0.26	47	265

Binding of aurovertin to Mg–ATP particles

The parameters of aurovertin binding to Mg–ATP particles were determined by measurement of the final fluorescence of bound aurovertin (i.e. at least pseudo-equilibrium conditions). The relative quantum yield (Q_{rel}) was calculated by extrapolation to infinite protein concentration, as described in the legends of Fig. 6 and Table I. Fig. 6 represents a Scatchard plot [15] for the binding of aurovertin to Mg–ATP particles oxidizing succinate under State-3 conditions. The results of three separate experiments are plotted together.

It can be seen that there are two binding sites for aurovertin, one with high affinity (concentration, n_1) and one with a lower affinity (concentration, n_2), and that binding of aurovertin to the first site is positively co-operative, the dissociation constant of the particle–aurovertin complex decreasing from 65 to 20 nM. Positive co-operative binding of aurovertin has also been shown for rat-liver mitochondria oxidizing succinate under State-3 conditions [10].

In Table I the binding parameters of aurovertin are listed under conditions of the experiment shown in Fig. 4, Trace A2. The first row of Table I shows that binding of aurovertin to Mg–ATP particles suspended in the standard medium, supplemented with 1 mM $MgCl_2$, occurs with a low relative quantum yield, while the ATPase enzyme exposes a high concentration of weak binding sites with a low affinity. Whereas phosphate has little effect on the binding parameters, a subsequent addition of ADP enhances the relative quantum yield by 50%.

The addition of succinate to complete State-3 conditions induces a conformation of F_1 that binds aurovertin with a high relative quantum yield, and the concentration of binding sites is decreased considerably, mainly the weak binding sites, that also have an increased affinity. The concentration of strong binding sites is increased, and aurovertin binding to this site becomes cooperative.

Upon anaerobiosis, cooperativity disappears, the relative quantum yield is diminished, and the binding parameters are comparable to those found in the absence of succinate, with the exception of a higher relative quantum yield. The binding data obtained after the addition of 1799 under aerobic conditions, followed by anaerobiosis, are comparable with those of endogenously uncoupled AS particles in the presence of $MgCl_2$, phosphate and ADP (Table III of ref. 12). The calculated aurovertin-binding parameters obtained immediately after the fast uncoupler-induced quenching or the oligomycin-induced enhancement of the fluorescence had been reached (Fig. 4, Trace A2) are not given, since it is doubtful whether even a pseudo-equilibrium exists under these conditions. The changes appeared, however, partly to be caused by a rapid change in the relative quantum yield (0.5 and 2.2 units/nM aurovertin, respectively).

DISCUSSION

The large enhancement of the fluorescence of aurovertin upon binding to F_1 is influenced by ligands of the enzyme, both in the isolated [6, 9, 11] and membrane-bound enzyme [8–10, 12]. In general, the changes in the fluorescence of the aurovertin-F_1 complex may be interpreted as being due to conformational changes of the enzyme, induced by the ligands, leading to an alteration of the aurovertin-binding sites. The results reported in this paper indicate that energization of the mitochondrial membrane

also changes the conformation of F_1 as monitored by the fluorescence of bound aurovertin.

We have shown that the ATPase inhibitor dissociates from Mg–ATP particles when oxidative energy is supplied, especially under State-3 conditions [4, 5]. A high ratio of ADP/ATP also induces a partial dissociation of the inhibitor [4]. Since we have reported that binding of the ATPase inhibitor to inhibitor-deficient submitochondrial particles or to isolated F_1 strongly quenches the aurovertin fluorescence, any enhancement of the fluorescence induced in the complex between aurovertin and Mg–ATP particles may be the result of three possible factors: (i) dissociation of the ATPase inhibitor; (ii) alteration in the binding of ligands to F_1; (iii) change of the energy state of the particles. Which of these factors causes the fluorescence changes described in this paper will be discussed.

The control experiments shown in Figs 1A, 1C and 1D indicate that the slow and extensive enhancement of the fluorescence of aurovertin that is induced by succinate oxidation (Fig. 1B) can be completely accounted for by dissociation of the ATPase inhibitor. This slow enhancement of the fluorescence is prevented not only by an uncoupler but also by oligomycin, indicating that the latter antibiotic prevents F_1 'feeling' energization of the membrane.

After the inhibitor has been dissociated from Mg–ATP particles during an incubation with succinate, the second factor becomes apparent. The resulting high aurovertin fluorescence is influenced by ligands of F_1 (Fig. 2). Phosphate and ATP (not shown) strongly quench it, while ADP has only a small effect, provided hexokinase is present. In this respect the effects of ligands are comparable to those induced in inhibitor-depleted AS particles [12] or isolated F_1 [9, 11].

The third factor, the energy state of the particles is directly felt by aurovertin only when all the State-3 components are present (Fig. 2). Measurements of the kinetics of the aurovertin fluorescence enhancement under State-3 conditions in oxygen-pulse experiments indicate that this change is very fast ($t_{\frac{1}{2}}$ about 20 ms). This value is comparable to half-times obtained for the initial phase in the NADH- or succinate-induced reduction of cytochromes *c*, *b* and *a* [16]. Half-times for oxidation of the succinate-reduced cytochromes *b* and c_1 in oxygen-pulse experiments in rat-liver mitochondria are in the order of 50–100 ms [17, 18]. Thus, the rate constants of conformational changes of F_1 induced by energization are comparable to those of rate-limiting redox changes of carriers in the respiratory chain.

The results of the aurovertin-binding experiments listed in Table I can be understood using the concepts developed in the two preceding papers [5, 12]. Two conformations of F_1 are in equilibrium, termed F_1, binding aurovertin in a highly fluorescent form and $*F_1$, binding the fluorochrome in a less fluorescent form. Conformation F_1 has only a strong aurovertin-binding site, while $*F_1$ is postulated to have both strong and weak sites. The equilibrium is shifted towards conformation $*F_1$ by binding of ATP or phosphate, whereas ADP induces conformation F_1 [12]. Moreover, binding of the ATPase inhibitor to inhibitor-depleted particles induces a shift from conformation F_1 to $*F_1$ [5].

When aurovertin binds to inhibitor-containing Mg–ATP particles the enzyme is in the low-fluorescent $*F_1$ conformation (Table I). Since phosphate induces the same conformation it has little effect. ADP elicits a partial dissociation of the ATPase inhibitor [4], giving rise to a higher fluorescence. When State-3 conditions are com-

plete (and the inhibitor has been dissociated [4]) a high fluorescence is obtained. This may be interpreted as a shift to conformation F_1. Binding of aurovertin to the strong site becomes cooperative, supporting the idea of the involvement of this site in the inhibition of oxidative phosphorylation [9, 12]. Upon anaerobiosis cooperativity disappears, the membrane de-energizes, concomitant with a shift back to the $*F_1$ conformation.

Effect of an uncoupler and oligomycin

The extent of the fast quenching of the fluorescence of aurovertin induced by an uncoupler seems to be dependent on the availability of ATP (Fig. 4). When no adenine nucleotides are present, the quenching is slow and slight (Fig. 1, Trace B). This suggests that the uncoupler induces a rapid association of the enzyme with ATP. This may be interpreted as a rapid shift to the $*F_1$ conformation (the quantum yield decreased by 70%). This result may support the proposal made by Boyer et al. [19] and Slater [20] that the energy-conserving step in oxidative phosphorylation is a conformational change of F_1, from a state where ATP is tightly bound to one where ATP is loosely bound. The slower rise in the aurovertin fluorescence following the fast quenching induced by an uncoupler (Fig. 4) may be due to a redistribution of bound adenine nucleotides, i.e. ATP is replaced by ADP, giving rise to a higher fluorescence (in Traces A and B of Fig. 4, substrate amounts of ADP were present). Because of the addition of an uncoupler the Mg–ATP particles are uncoupled, and in this case the ATP may not be tightly bound and can be replaced by ADP.

This interpretation of the effect of an uncoupler is supported by the fact that oligomycin prevents the fast quenching induced by an uncoupler (Trace A2 of Fig. 4) (oligomycin prevents binding of ATP to the particles and replacement of a ligand by ADP [12]).

The oligomycin-induced enhancement of the aurovertin fluorescence (Fig. 5) may be explained in the following way. The more coupled the membrane is, the higher the aurovertin fluorescence under State-3 conditions. Oligomycin is also able to restore coupling of partially or completely uncoupled submitochondrial particles [13, 21, 22]. Fig. 5 shows that the oligomycin effect is small at a low pH and large at a high pH. Since a low pH induces a better coupled submitochondrial membrane [13, 14], the results of Fig. 5 suggest that addition of oligomycin to Mg–ATP rapidly induces a maximally coupled membrane. This conclusion is in line with the extent of the uncoupler-induced quenching of the fluorescence at different pH values (Fig. 5).

ACKNOWLEDGEMENTS

We thank Mr P. J. Sondervan for his excellent technical assistance and Dr R. M. Bertina for helpful discussions. We wish to thank Professor Dr E. C. Slater for his helpful and stimulating discussions throughout this investigation. We are grateful to Mrs Katja van de Stadt for her help in preparing the manuscript. This work was supported in part by The Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

REFERENCES

1 Löw, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 361–374
2 Pullman, M. E. and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762–3769
3 Horstman, L. L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344
4 Van de Stadt, R. J., De Boer, B. L. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 338–349
5 Van de Stadt, R. J. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 240–252
6 Lardy, H. and Lin, C. H. C. (1969) in Inhibitors—Tools in Cell Research (Bücher, Th. and Sies, H., eds) pp. 279–281, Springer Verlag, Berlin
7 Lardy, H. A., Connelly, J. L. and Johnson, D. (1964) Biochemistry 3, 1961–1968
8 Bertina, R. M. (1972) The Interaction of Oligomycin and Aurovertin with the ATPase Complex in Intact Mitochondria, Ph. D. thesis, University of Amsterdam, Gerja, Waarland
9 Chang, T. and Penefsky, H. S. (1973) J. Biol. Chem. 248, 2746–2754
10 Bertina, R. M., Schrier, P. I. and Slater, E. C. (1973) Biochim. Biophys. Acta 305, 503–518
11 Yeates, R. A. (1974) Biochim. Biophys. Acta, 333, 173–179
12 Van de Stadt, R. J., Van Dam, K. and Slater, E. C. (1974) Biochim. Biophys. Acta 347, 224–239
13 Groot, G. S. P. (1970) Enige Aspecten van de Mitochondriale Energiehuishouding, Ph. D. thesis, University of Amsterdam, Mondeel, Amsterdam
14 Hinkle, P. C. and Horstman, L. L. (1971) J. Biol. Chem. 246, 6024–6028
15 Scatchard, G., Scheinberg, I. H. and Armstrong, Jr, S. H. (1950) J. Am. Chem. Soc. 72, 535–540
16 Lee, C. P., Ernster, L. and Chance, B. (1969) Eur. J. Biochem. 8, 153–163
17 Chance, B., Azzi, A., Lee, I. Y., Lee, C. P. and Mela, L. (1969) in Mitochondria, Structure and Function (Ernster, L. and Drahota, Z., eds), FEBS Symp., Vol. 17, pp. 233–273, Academic Press, New York
18 Chance, B., Lee, C. P., Lee, I. Y., Ohnishi, T. and Higgins, J. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 29–59, Adriatica Editrice, Bari
19 Boyer, P. D., Cross, R. L. and Momsen, W. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2837–2839
20 Slater, E. C. (1974) in Dynamics of Energy Transducing Membranes (Ernster, L., Estabrook, R. W. and Slater, E. C., eds), Elsevier, Amsterdam, in the press.
21 Lee, C. P. and Ernster, L. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), BBA Library, Vol. 7, pp. 218–234, Elsevier, Amsterdam
22 Papa, S., Guerrieri, F., Rossi Bernardi, L. and Tager, J. M. (1970) Biochim. Biophys. Acta 197, 100–103