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THE BINDING OF AUROVERTIN TO MITOCHONDRIA, AND ITS EFFECT ON MITOCHONDRIAL RESPIRATION

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SUMMARY

1. The fluorescence of aurovertin increases about 100-fold on binding to sub-mitochondrial particles.

2. The mitochondrial ATPase (F_1) binds one mole aurovertin/mole F_1 with a dissociation constant of $6 \cdot 10^{-8}$ M.

3. The fluorescence of mitochondrion-bound aurovertin is maximal during State-3 respiration and is partially quenched on anaerobiosis, addition of respiratory inhibitor, oligomycin or uncoupler, or transition to State 4. This quenching is still present when the binding site is saturated with aurovertin, showing that the quantum yield of fluorescence is lowered.

4. Aurovertin is bound co-operatively to State-3 mitochondria.

5. The curve relating inhibition of State-3 respiration to aurovertin concentration is more sharply sigmoidal than the binding curve.

6. An analysis of the binding and inhibition data leads to the conclusion that aurovertin induces a conformation change in the binding site on F_1 in two ways: (i) directly by acting as an allosteric effector of an oligomeric system, (ii) indirectly by inhibiting State-3 respiration which changes the allosteric constant of the oligomeric system.

7. The concentration of the aurovertin-binding site in both rat-liver and rat-heart mitochondria is about the same as that of the antimycin-binding and oligomycin-binding sites.

INTRODUCTION

Aurovertin, introduced by Lardy[1] in 1960, is as effective an inhibitor of oxidative phosphorylation as oligomycin. It is, however, much less active than oligomycin in inhibiting ATP-driven reactions catalysed by mitochondrial preparations[2–6]. Lee and Ernster[4] proposed that it inhibits the transfer of phosphate from $X \sim P$, a hypothetical high-energy phosphate intermediate, to ADP by competition with $X \sim P$. Lardy and Lin[7] have shown that it binds specifically to isolated mitochondrial ATPase (coupling factor F_1, ref. 8).

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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Aurovertin resembles oligomycin in that the curve relating inhibition of oxidative phosphorylation with concentration of inhibitor is sigmoidal[2,3,5] whereas the corresponding inhibitor–effect curves for the ATP–P_i and ATP–ADP exchange reactions are hyperbolic[2,5]. As an explanation of the different inhibitor–effect curves found with oligomycin, we have proposed as a working hypothesis that oligomycin combines preferentially with a conformation of the membrane that is present in higher concentrations in energized mitochondria than in non-energized[6,9,10]. It is difficult to test this hypothesis further, in the absence of a convenient method for directly measuring the binding of oligomycin to the membrane. In this respect, aurovertin is more promising since the aurovertin–F_1 complex is strongly fluorescent[7]. Although it is clear that the binding site of aurovertin[7] is different from that of oligomycin[11], it seemed more profitable to study the former inhibitor, since the change in fluorescence on binding of aurovertin to the membrane enables studies similar to those already reported for antimycin, which has also been proposed to bind preferentially to energized membranes[10,12].

Furthermore, experience with anilinonaphthalene sulphonate as a fluorescent probe for enzymes[13] suggests that aurovertin, specifically bound to F_1 in the mitochondrial membrane, might prove a useful probe for any conformational changes in F_1 accompanying changes in the energy state of the membrane.

METHODS AND MATERIALS

Rat-liver mitochondria were isolated according to the method of Hoogeboom[14] as described by Myers and Slater[15]. Rat-heart mitochondria were isolated according to a modification (ter Welle, H. F., personal communication) of the method described by Tyler and Gonze[16].

Mitochondrial ATPase (F_1) was isolated as described by Horstman and Racker[17].

Protein was determined by the biuret method as described by Cleland and Slater[18].

Fluorescence spectra of aurovertin were measured at 25 °C under continuous stirring in a Perkin–Elmer MPF 2A fluorimeter using the front-face method. The slit width for both excitation and emission spectra was 4 nm.

The binding of aurovertin to mitochondrial preparations was measured in an Eppendorf fluorimeter with excitation filter 313+366 nm and emission filter 470–3000 nm. A closed thermostated cuvette (capacity 1 ml) fitted with a Clark oxygen electrode was used, so that fluorescence and oxygen uptake could be measured simultaneously.

State-3 respiration was measured polarographically at 25 °C in a medium containing 15 mM KCl, 25 mM Tris–HCl buffer, 2 mM EDTA, 5 mM $MgCl_2$, 100 mM sucrose, 10 mM P_i and 10 mM succinate. The pH was 7.4. 0.2–0.6 μg rotenone/mg protein was also present. State-3 respiration was started by the addition of 1–2 mM ADP.

The concentration of aurovertin solutions was measured spectrophotometrically at 367.5 nm using an absorbance coefficient of 42.7 $mM^{-1} \cdot cm^{-1}$ (ref. 19).

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of Dr P. G. Heytler, atractyloside of Prof. V. Sprio, oligomycin of the Upjohn Chemical

Company and aurovertin of Dr H. Lardy. Rotenone and antimycin were obtained from Penick and Co. and Sigma, respectively.

RESULTS

Fluorescence spectra of aurovertin

Although Lardy and Lin[7] reported that aurovertin exhibits no fluorescence in either aqueous or non-polar solvents, typical emission and excitation spectra can be measured with sufficiently concentrated solutions (Fig. 1, dashed lines). The excitation spectrum reveals two maxima, at 277 nm and 370 nm, respectively, that are close to those in the ultraviolet-absorption spectrum, *viz.* at 270 nm and 367.5 nm[19], with a shoulder at 393 nm, The emission spectrum shows a double maximum at 470 nm and 492 nm, when 368 nm is used as excitation wavelength. Fig. 1 (full lines) shows the fluorescence spectra of aurovertin in the presence of A particles. It appears that the maxima in the excitation spectrum have been moved

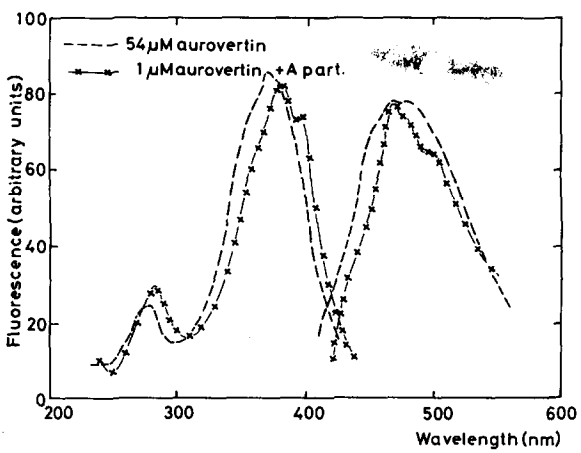


Fig. 1. Uncorrected fluorescence spectrum of 54 μM aqueous aurovertin (– – – –). The excitation spectrum (left) was measured at an emission wavelength of 480 nm, the emission spectrum (right) at an excitation wavelength of 368 nm. Medium: 10 mM Tris–HCl buffer (pH 7.4), 250 mM sucrose. Uncorrected fluorescence spectrum of 1 μM aurovertin in the presence of 1 mg 'A' particles (× — ×). The spectra are corrected for the emission and excitation of 'A' particles in the absence of aurovertin. Other conditions as in absence of particles.

to slightly higher wavelengths. The fluorescence is plotted in the same arbitrary units as in the absence of particles, although the concentration of aurovertin is only one fiftieth. From the two emission spectra it can be calculated that the fluorescence yield of particle-bound aurovertin is at least 54 times higher than that of free aurovertin. Because of the excess of aurovertin present in this experiment this is only a minimum value. Experiments at lower aurovertin concentrations show that the increase in fluorescence yield is at least 100-fold.

Binding of aurovertin to beef-heart F_1

Fig. 2 shows the increase in fluorescence at 470–3000 nm due to the addition

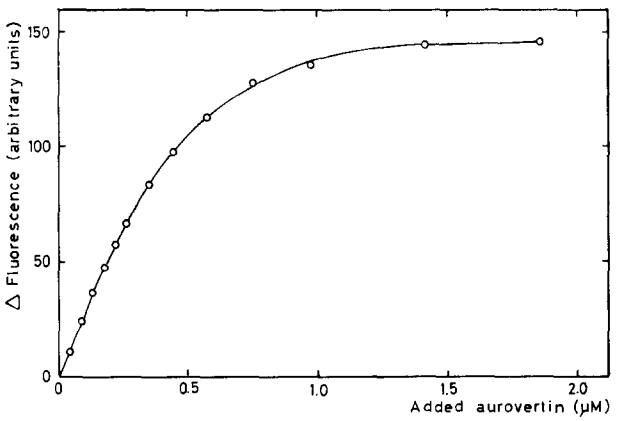


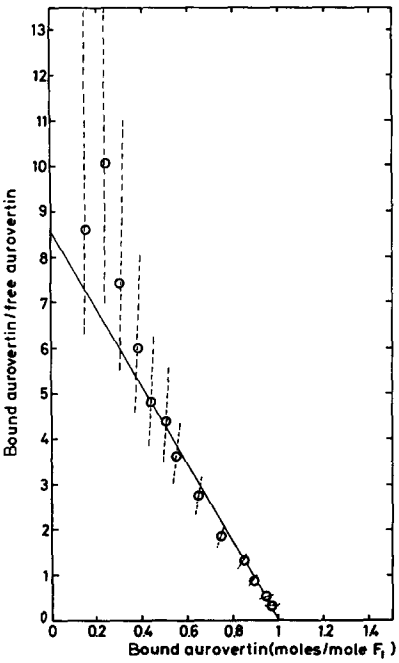
Fig. 2. The binding of aurovertin to F_1. The increase in fluorescence caused by the addition of various concentrations of aurovertin to 0.5 μM F_1 dissolved in the medium used for measuring State-3 respiration (see Methods) at 25 °C.

of different amounts of aurovertin to a 0.5 μM solution of F_1 (mol. wt 360000, ref. 20). The number of aurovertin-binding sites and the dissociation constant (K_D) of the aurovertin–F_1 complex can be calculated if the following assumptions are made: (i) there is a linear relationship between the increase in fluorescence and the amount of aurovertin bound; (ii) free aurovertin does not contribute to the increase in fluorescence. A good fit of the experimental with the calculated points was obtained if 0.5 μM bound aurovertin corresponds with 150 units. The Scatchard plot shown in Fig. 3 shows that one aurovertin-binding site is present per molecule of F_1. This is in agreement with the conclusion of Lardy and Lin[7], who also found a 1:1 stoicheiometry, but they used a molecular weight for F_1 of 284000. The K_D of the aurovertin–F_1 complex was calculated from the straight line, shown in Fig. 3, to be $6 \cdot 10^{-8}$ M. The accuracy of the experimental data does not permit any conclusion as to the existence of negative co-operativity or inhomogeneity in binding sites (F_1). A Hill plot of the experimental data shown in Fig. 3 yields a completely straight line with a slope of 0.9.

Binding of aurovertin to intact mitochondria

Since NAD(P)H fluoresces at the wavelengths used for studying the fluorescence of aurovertin, it was necessary to consider whether changes in mitochondrial NAD(P)H contribute significantly to the fluorescence measured in the presence of aurovertin under the experimental conditions employed (rotenone was present). At the sensitivity used in these experiments, no significant change in fluorescence in the absence of aurovertin was observed under conditions in which it is known that there may be large changes in the concentration of NAD(P)H (transition from State 3 to State 4, inhibition of State-3 respiration by antimycin or oligomycin, or anaerobiosis). Thus it may be concluded that changes in the concentration of NAD(P)H do not contribute appreciably to changes in the fluorescence measured under the experimental conditions used.

Fig. 4 shows the effect of adding aurovertin to State-3 mitochondria. As is



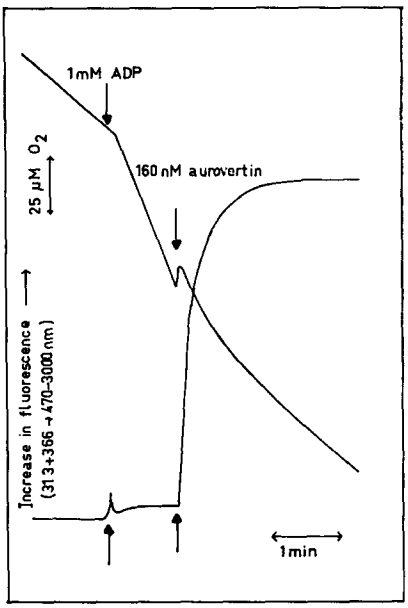


Fig. 3. Scatchard plot for the binding of aurovertin to F_1. The concentrations of F_1-bound and free aurovertin were calculated from the data in Fig. 2, taking 150 units as equivalent to 500 nM bound aurovertin. The dotted lines show the effect of a 2% error in the concentration of added and bound aurovertin on the experimental points in the Scatchard plot.

Fig. 4. Binding of aurovertin to State-3 mitochondria. Fluorescence and O_2 consumption were measured simultaneously. Volume, 1 ml; pH 7.4, 25 °C; 0.36 mg rat-heart mitochondria; State-3 succinate oxidation medium (see Methods).

also the case with isolated F_1, the increase in fluorescence is biphasic. The first rapid phase is not accompanied by any measurable inhibition of the respiration, while the second slower phase parallels the progressive inhibition. A constant fluorescence is reached when the partially inhibited respiratory rate is also constant. The final increase in fluorescence was used as a measure for the equilibrium distribution of aurovertin over particles and solution. In Fig. 5 it is shown that the fluorescence is quenched when ATP synthesis is inhibited by (i) anaerobiosis (A) or addition of a respiratory-chain inhibitor (B), (ii) addition of oligomycin (C) or (iii) transition from State 3 to State 4 (D). The decrease brought about by addition of oligomycin is not released by addition of uncoupler (Fig. 6A), whereas the addition of uncoupler alone to State-3 mitochondria markedly decreases the aurovertin fluorescence (Fig. 6B). Addition of oligomycin after anaerobiosis causes a further quenching of the fluorescence (Figs 5A and 5D). This does not seem to be due to competition between oligomycin and aurovertin for the same binding site, since the addition of much larger concentrations of oligomycin did not quench the fluorescence any further.

It may be concluded that maximum fluorescence of F_1-bound aurovertin in mitochondria is obtained under conditions of uninhibited energy transfer into the F_1

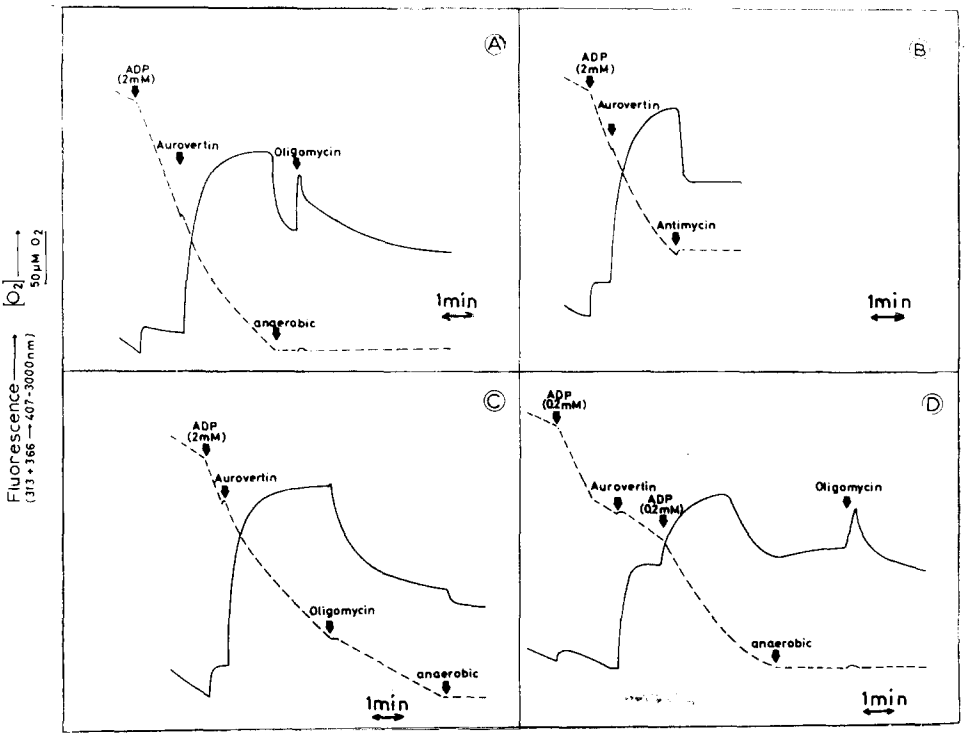


Fig. 5. Fluorescence of mitochondrion-bound aurovertin under various conditions. Volume 1 ml; pH 7.4, 25 °C; 0.5 mg rat-liver mitochondria. State-3 succinate oxidation medium. The fluorescence of mitochondrion-bound aurovertin (0.042 μg) was followed through the following transitions: (A) State 3–anaerobiosis–*plus* oligomycin (0.4 μg); (B) State 3–*plus* antimycin (0.05 μg); (C) State 3–*plus* oligomycin (0.4 μg)–anaerobiosis; (D) State 4–State 3–State 4–anaerobiosis–*plus* oligomycin (0.4 μg).

region and a high ADP/ATP ratio (State 3). The quenching of the fluorescence that occurs by inhibition of energy transfer or at low ADP/ATP ratios could be due to either (a) a decrease in the quantum yield of the fluorescence, (b) an increase in the dissociation constant of the protein–ligand complex, (c) a decrease in the number of binding sites, or (d) inner-filter quenching. It seems unlikely, however, that inner-filter quenching plays an important role, since addition of uncoupler to oligomycin-inhibited mitochondria (Fig. 6A) or of antimycin to or removal of oxygen from respiring non-phosphorylating sub-mitochondrial particles, treatments leading to large changes in the redox state of respiratory carriers, have no effect on the fluorescence of the particle-bound aurovertin.

Brocklehurst *et al.*[21] have described a method in which it is possible to differentiate between effects on the quantum yield of the protein-bound fluorochrome and on the binding parameters. If measurements are carried out at constant concentration of fluorochrome and variable protein concentration, extrapolation to infinite protein concentration reveals the situation when all the ligand is bound to the protein. Fig. 7A shows double-reciprocal plots of the fluorescence and the protein concentration at a fixed concentration of aurovertin. Even at infinite protein concen-

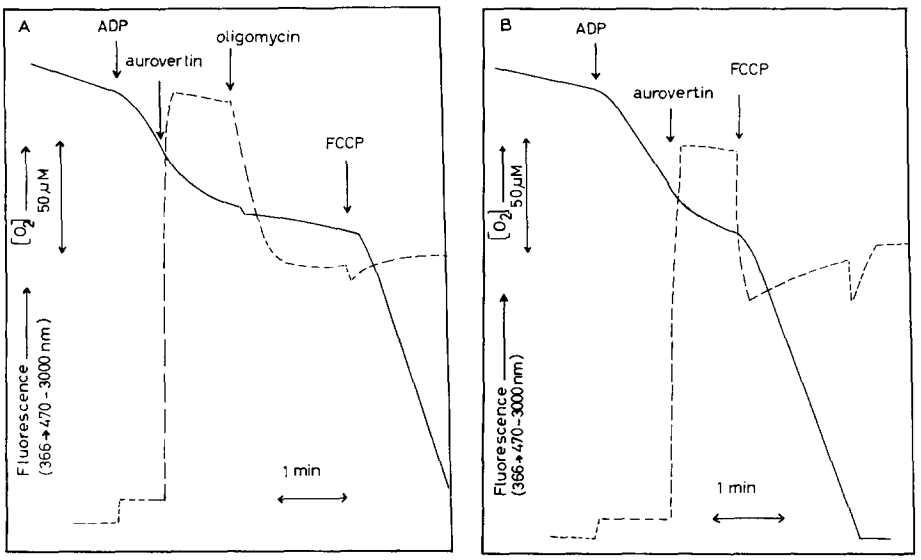


Fig. 6. Effect of uncoupler on the aurovertin fluorescence. Volume, 1.7 ml; pH 7.4, 25 °C; State-3 succinate oxidation medium (see Methods); 0.5 mg rat-liver mitochondria, 0.25 μM FCCP, 0.75 μg aurovertin, 0.7 μg oligomycin. The fluorescence of mitochondrion-bound aurovertin was followed through the following transitions: (A) State 3–*plus* oligomycin–*plus* uncoupler; (B) State 3–*plus* uncoupler.

tration, antimycin still quenches the fluorescence. The difference in the intersection points of the ordinate in Fig. 7A is significant at a level of $P=0.06$. Since similar results were obtained in a number of experiments, it is clear that the difference is well outside the experimental error. This is also illustrated by the points shown in Fig. 7B which indicates that, independent of the aurovertin concentration, antimycin quenches the fluorescence by 30%. The straight lines in Fig. 7B show that, both in the presence and absence of antimycin, the fluorescence yield of mitochondria-bound aurovertin is independent of the degree of saturation of the mitochondria with aurovertin. Similar plots are obtained when the respiration is stopped by anaerobiosis.

The binding of aurovertin to State-3 mitochondria

In Fig. 8 the maximum increase in fluorescence observed after addition of aurovertin to State-3 mitochondria is plotted against the concentration af added aurovertin. The curve shows that at increasing aurovertin concentrations the overall affinity of aurovertin for its receptor site increases. At each concentration of added aurovertin, the concentration of bound and free ligand may be calculated from the observed fluorescence and the fluorescence of bound aurovertin, determined by extrapolation to infinite protein concentration at a single aurovertin concentration, as described in Fig. 7. In Fig. 9 the experimental data of Fig. 8, calculated in this way, are plotted as a Scatchard plot, from which it can be calculated that the number of aurovertin-binding sites equals 0.27 nmole/mg rat-heart mitochondria and that the final K_D of the aurovertin–F_1 complex is 25 nM.

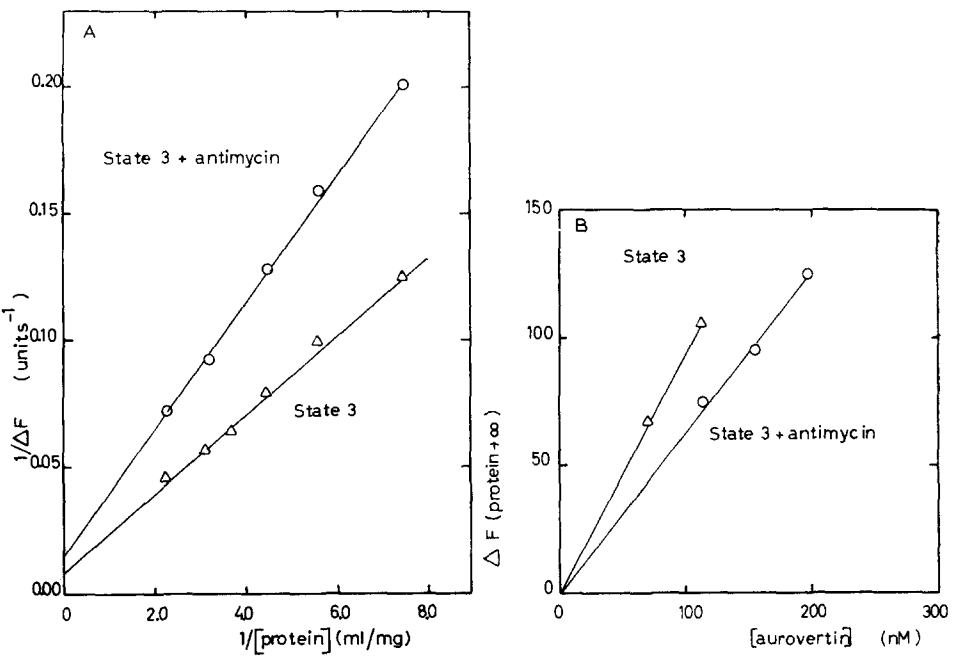


Fig. 7. Influence of antimycin on the fluorescence yield of mitochondrion-bound aurovertin: (A) $1/\Delta F$ plotted against 1/[protein]. Antimycin, 0.13 μg; aurovertin, 80 nM; State-3 succinate oxidation medium (see Methods); rat-liver mitochondria. Volume, 1.6 ml; pH 7.4, 25 °C. $(1/\Delta F)_{\text{protein}=\infty}$ is 0.84 ($\pm$0.12 (S.E.)) in the absence of antimycin and 1.39 ($\pm$0.16) in the presence of antimycin. (B) $\Delta F_{\text{protein}=\infty}$ plotted against aurovertin concentration in the presence or absence of antimycin. Antimycin, 0.13 μg; State-3 succinate oxidation medium (see Methods); rat-liver mitochondria. Volume, 1.65 ml; pH 7.4, 25 °C.

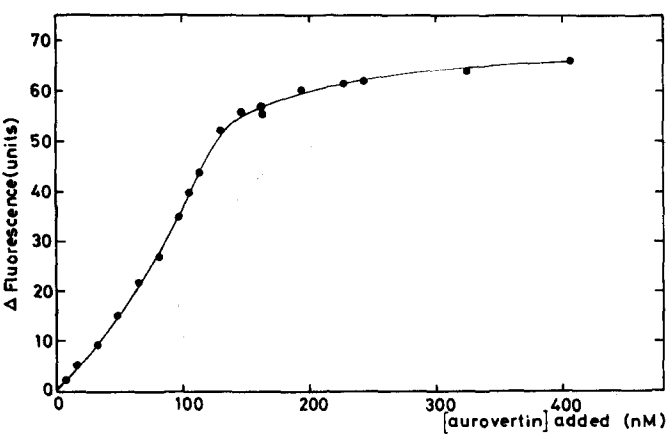


Fig. 8. Binding of aurovertin to State-3 rat-heart mitochondria. The experiments were carried out as in Fig. 4. The final increase in fluorescence is plotted against the added aurovertin concentration. Succinate was used as a substrate, in the presence of rotenone. Volume, 1.0 ml; pH 7.4, 25 °C; 0.18 mg rat-heart mitochondria.

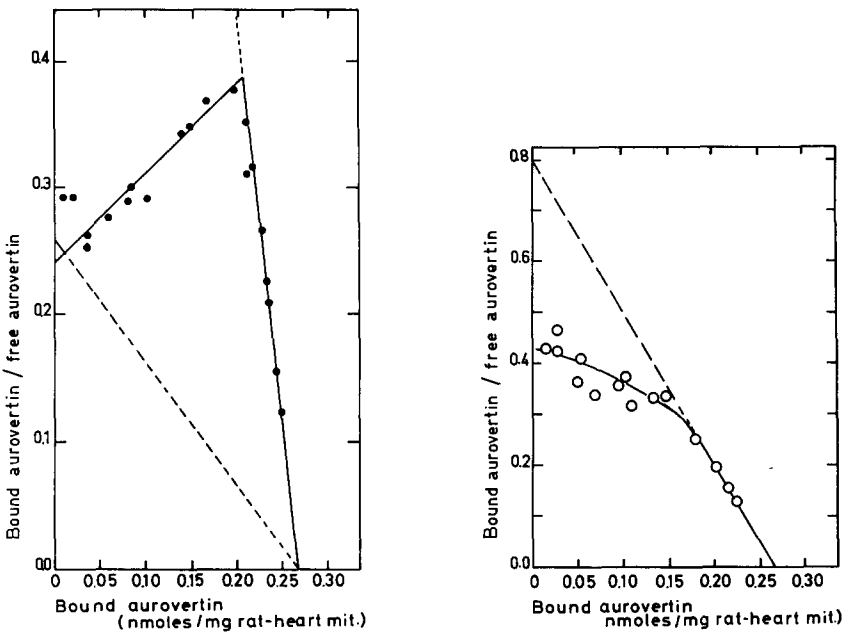


Fig. 9. Scatchard plot for the binding of aurovertin to State-3 heart mitochondria, calculated from the data shown in Fig. 8. Bound and free aurovertin concentrations were calculated from the fluorescence yield of mitochondrion-bound aurovertin (1480 units·μM^{-1}) determined by extrapolation to infinite protein concentration as described in Fig. 7A. From the dotted lines the affinity of aurovertin for State-3 mitochondria at zero aurovertin concentration and at saturating aurovertin concentration can be estimated (see Discussion).

Fig. 10. Scatchard plot for the binding of aurovertin to anaerobic State-3 rat-heart mitochondria. Experimental conditions were identical to those of Fig. 8, except that the increase in fluorescence was measured after anaerobiosis. The fluorescence yield of mitochondrion-bound aurovertin was found to be 1125 units·μM^{-1}.

Fig. 9 shows that there is a strong co-operativity in the binding of aurovertin to State-3 mitochondria. Since aurovertin inhibits State-3 respiration, the co-operativity may reflect an increased affinity of aurovertin for aurovertin-inhibited mitochondria, rather than allosteric binding of aurovertin *per se*.

The binding of aurovertin to anaerobic mitochondria is also somewhat co-operative (Fig. 10). Once again, this may represent a stronger binding to aurovertin-inhibited mitochondria rather than a true allosteric effect. F_1 is still turning over under these conditions, since mitochondria catalyse an ATP–P_i exchange. A comparison of Figs 9 and 10 shows that anaerobiosis has no effect on the concentration of binding sites, but causes an increase in the dissociation constant of the mitochondria–F_1 complex from 25 to 60 nM.

Inhibition of State-3 respiration by aurovertin

Fig. 11 shows that the aurovertin–inhibition curve does not coincide with the aurovertin–binding curve. When 50% of the binding sites are occupied by aurovertin, respiration is inhibited by less than 20%. On the other hand, maximal in-

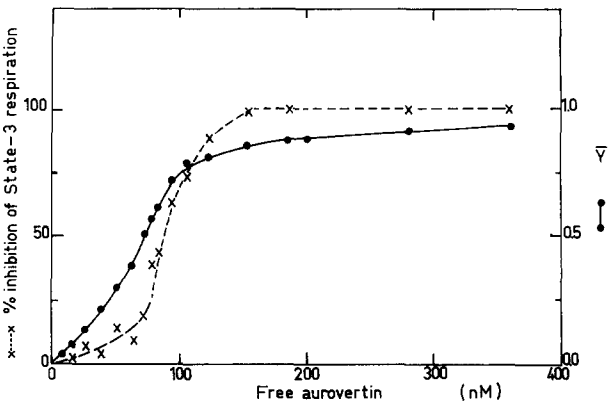


Fig. 11. Aurovertin binding and inhibition as function of free aurovertin concentration. Binding and inhibition data are derived from the experiment shown in Fig. 8. The percentage of inhibition of State-3 respiration was calculated after correction for the aurovertin-insensitive "State-4" respiration. $\bar{Y}$ is the fraction of binding sites occupied by aurovertin.

hibition is obtained when only about 85% of the sites are occupied. Before these observations can be discussed properly, it is necessary to discuss a simple kinetic explanation for the occurrence of sigmoidal inhibition curves.

The sigmoidal curve relating inhibition of State-3 respiration to aurovertin concentration[2,5] might be simply explained if the maximal turnover of the aurovertin-sensitive site (*i.e.* the phosphorylation system) is much higher than that of the reaction that limits State-3 respiration, and a distributive centre is present between the phosphorylating system and the system that catalyses this rate-limiting reaction. The fact that the rate of oxidation of succinate by rat-heart mitochondria equals that of glutamate, although the P:O ratio is 50% higher with the latter substrate, suggests that electron transport is the rate-limiting reaction in rat-heart mitochondria. Kemp *et al.*[22] have shown that the rate-limiting reaction for State-3 respiration in rat-liver mitochondria, where essentially the same results are obtained for aurovertin binding and inhibition as in rat-heart mitochondria, is the transport of ADP into the mitochondria. If distributive centres were present between electron transfer and phosphorylation or between phosphorylation and ADP transport, one might expect that partial inhibition of phosphorylation by aurovertin would bring about sigmoidal effect curves describing the effect of inhibitors on electron transfer or ADP transport. Figs 12A and 12B show that this is not found with NH_2OH as an inhibitor of electron transfer or atractyloside as inhibitor of ADP transport. Moreover, partial inhibition of electron transfer by either NH_2OH or antimycin does not result in sigmoidal atractyloside–effect curves (Figs 12C and 12D), although inhibition by malonate does (Fig. 12E). There is, then, evidence for a distribution centre between succinate dehydrogenase and ADP transport (maybe ubiquinone[23]), but not between the electron-transfer chain and ADP transport. It appears unlikely, then, that the sigmoidicity of the aurovertin–inhibition curve (shown in Fig. 11), is partially the result of a relatively high turnover of the aurovertin-sensitive site in the way discussed above. A qualitative and quantitative model for the aurovertin binding and inhibition data (see Fig. 11) will be proposed in the discussion.

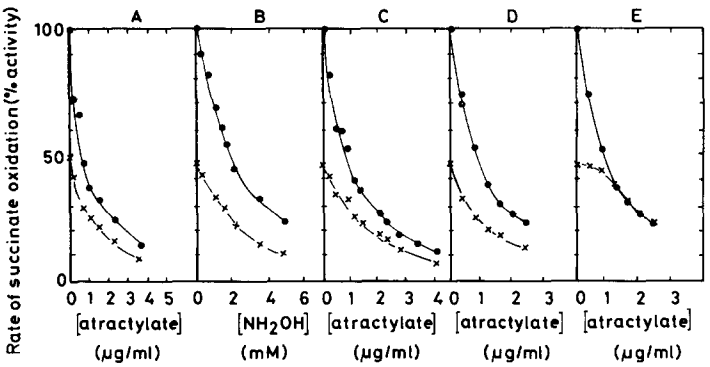


Fig. 12. Effect of combinations of inhibitors on State-3 oxidation of succinate. State-3 succinate oxidation was measured as described under Methods. The State-3 respiration rate in the absence of any inhibitor is referred to as 100%. Total volume, 1.75 ml. (A) ●—●, no additions; ×—×, partially inhibited by aurovertin. (B) ●—●, no additions; ×—×, partially inhibited by aurovertin. (C) ●—●, no additions; ×—×, partially inhibited by NH_2OH. (D) ●—●, no additions; ×—×, partially inhibited by antimycin. (E) ●—●, no additions; ×—×, partially inhibited by malonate.

DISCUSSION

The large enhancement of the aurovertin fluorescence that occurs upon binding to F_1 can be used for several purposes: (1) it provides a sensitive probe for conformational changes in the F_1 region of the mitochondrial inner membrane under phosphorylating conditions; (2) it can be used to titrate the F_1 content of various mitochondrial preparations, and (3) it allows aurovertin-binding curves to be determined even under turnover conditions.

The experiments shown in Figs 5 and 6 suggest that the fluorescence yield of mitochondrion-bound aurovertin monitors a specific conformation of the F_1–aurovertin complex, that is present in the highest concentration under conditions that favour ATP synthesis. Complete inhibition of electron transport or energy transfer or uncoupling of phosphorylation leads to a lower fluorescence yield.

These results are not in disagreement with the earlier report of Chance *et al.*[24] that the fluorescence of aurovertin is insensitive to oxidized-reduced or coupled–uncoupled transitions, since the quenching of aurovertin fluorescence is obtained only under State-3 conditions (not tested by Chance *et al.*[24]). The chemical event that is responsible for the quenching of the aurovertin fluorescence is difficult to identify because of the localization of F_1 in an inner membrane that separates inner and outer compartments of different chemical composition. In experiments not shown here it was observed for instance that the concentration of ATP, Mg^{2+}, P_i or H^+ all affect the fluorescence yield of aurovertin bound to submitochondrial 'A' particles.

Table I shows that the concentration of the aurovertin-binding site in both rat-liver and rat-heart mitochondria is about the same as that of the antimycin-binding and oligomycin-binding sites. The concentration of the latter two sites was calculated from the effects of the two inhibitors on State-3 respiration. This is justified, in view of the very low dissociation constants of the antimycin[12] and oligomycin

TABLE I

STOICHEIOMETRY OF AUROVERTIN-, ANTIMYCIN- AND OLIGOMYCIN-BINDING SITES

The concentrations were determined as follows (see also text): for oligomycin from the titre of oligomycin for the inhibition of State-3 respiration (measured 10 min after addition of oligomycin to State-3 mitochondria, when a constant rate of oxidation has been reached) and the P_i–ATP exchange (after 3-min preincubation with the inhibitor); for antimycin from the titre of the inhibitor for inhibition of State-3 respiration; for aurovertin from aurovertin-binding data (see Table II).

Mitochondria	*Concentration (μmole/g protein)*		
	Antimycin	*Oligomycin*	*Aurovertin*
Rat heart	0.26	0.27	0.27
Rat liver	0.10	0.12	0.12

(unpublished) complexes. The equivalence of the concentrations of the three binding sites suggests that there is a stoicheiometric relationship between the components of the electron-transfer chain and of the ATPase complex (consisting of both F_1 and the oligomycin-binding site on CF_0[11]).

The binding of aurovertin to mitochondria, but not to isolated F_1, is co-operative in the sense that addition of aurovertin in subsaturating amounts induces a conformation of F_1 with a higher binding constant for aurovertin. In general, this could be brought about in two ways: (1) binding of aurovertin to one molecule of F_1 in an oligomeric system induces a conformation change in other molecules of F_1 in the oligomer; (2) inhibition of State-3 respiration by aurovertin induces a conformation change in F_1. This second possibility is excluded by the fact that inhibition is appreciable only after more than about one half of the aurovertin-binding sites are occupied (Fig. 12). The inhibition curve is much more sigmoidal than the binding curve and the two cross at about 75% sutaration. Thus, binding of aurovertin cannot in itself cause inhibition of State-3 respiration, but it induces a change to an inhibited conformation of the phosphorylating system.

A minimum hypothesis summarizing these properties is given by the equations

$$\mathrm{R} \rightleftharpoons \mathrm{T} \qquad L = \frac{[\mathrm{T}]}{[\mathrm{R}]}$$

$$\mathrm{R} + \mathrm{A} \rightleftharpoons \mathrm{RA} \qquad K_\mathrm{R} = \frac{[\mathrm{R}]\cdot[\mathrm{A}]}{[\mathrm{RA}]} \qquad \alpha = \frac{[\mathrm{A}]}{K_\mathrm{R}}$$

$$\mathrm{T} + \mathrm{A} \rightleftharpoons \mathrm{TA} \qquad K_\mathrm{T} = \frac{[\mathrm{T}]\cdot[\mathrm{A}]}{[\mathrm{TA}]}$$

in which R represents the catalytically inactive conformation. Since the observed dissociation constant calculated from the Scatchard plot increases with increasing aurovertin concentration, and the degree of inhibition increases sigmoidally, K_R must be less than K_T, *i.e.* $c = (K_\mathrm{R}/K_\mathrm{T}) < 1$. The saturation function

$$\bar{Y}=\frac{\alpha(\ +Lc)}{1+L+\alpha(1+Lc)}$$

is a sigmoidal function of α only if L decreases with increasing aurovertin concentration.

$\bar{Y}$ is also a sigmoidal function of α, at constant L, if there is an oligomeric organization of n interacting F_1 molecules, according to the model of Monod *et al.*[25]. In this case,

$$\bar{Y}=\frac{\alpha(1+\alpha)^{n-1}+L\alpha c(1+\alpha c)^{n-1}}{(1+\alpha)^n+L(1+\alpha c)^n}$$

In analogy to Horn and Börnig[26], this may be written in the form

$$\log\left(\frac{\bar{Y}}{\alpha-(1+\alpha)\bar{Y}}-\frac{c}{1-c}\right)=(n-1)\{\log(1+\alpha)-\log(1+\alpha c)\}-\log(1-c)L \quad (1)$$

If L_0 (the value of L at zero aurovertin concentration) is $\geqslant 50$, the values of K_D measured from the Scatchard plot at zero and saturating concentrations of aurovertin, respectively, approximate K_R and K_T, respectively. Using these values calculated from Fig. 9 and the values of $\bar{Y}$ shown in Fig. 12, the left-hand side of Eqn 1 has been plotted against $\log(1+\alpha)-\log(1+\alpha c)$ in Fig. 13 (blocked circles). Instead of the straight line expected according to Eqn 1, two straight lines of about the same slope are obtained at both low and high aurovertin concentrations, separated by a region in which the allosteric constant L, calculated from the intercept of the straight lines with the ordinate, changes from 72 at low aurovertin concentrations to 2.4 at high. From the slope of the two parallel lines (equal to $n-1$), a value of

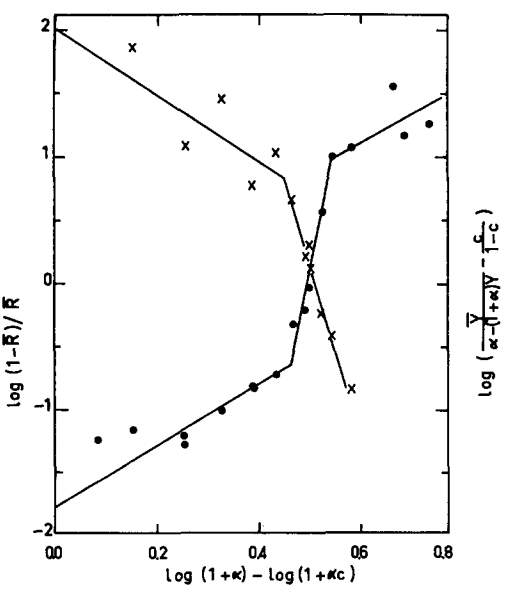


Fig. 13. Aurovertin-binding and -inhibition data from Fig. 12 analysed according to Eqns 1 and 2, respectively.

$n=3.4$ may be calculated. There is no evidence that, at any aurovertin concentration, $n=1$ (slope $=0$), which suggests that the binding site is oligomeric.

If, as suggested above, the T state is the catalytically active state and inhibition is caused by conversion to the R state, then the relation between $\bar{R}$, the fraction in the R state, and the degree of inhibition ($\beta=\%$ inhibition/100) is given by

$$\bar{R} = \frac{1+\beta L_0}{1+L_0}$$

where L_0 is the value of L in the absence of aurovertin. If $L \geqslant 50$, $\bar{R}=\beta$.

According to the model of Monod,

$$\bar{R} = \frac{(1+\alpha)^n}{L(1+\alpha c)^n+(1+\alpha)^n}$$

which may be transformed into the form

$$\log \frac{1-\bar{R}}{\bar{R}} = -n\{\log(1+\alpha)-\log(1+c\alpha)\}+\log L$$

Fig. 13 shows also the inhibition data plotted according to this equation. At low aurovertin concentrations, a line is obtained from which L may be calculated to be 100 and $n=3.0$, quite similar to those calculated from the $\bar{Y}$ function at these concentrations. A sharp change of slope is seen at the same concentration where the change in $\bar{Y}$ function also occurs.

Essentially the same results were found with rat-liver mitochondria. The calculated values of the parameters of the Monod model are assembled in Table II.

TABLE II

PARAMETERS FOR THE BINDING OF AUROVERTIN TO MITOCHONDRIA UNDER CONDITIONS OF STATE-3 RESPIRATION

See text for description of parameters.

	Rat heart	*Rat liver*
Number of binding sites (μmole/g protein)	0.27	0.12
K_T (nM)	216	115
K_R (nM)	24.6	9.2
c (K_R/K_T)	0.11	0.08
Analysis of $\bar{R}$ function		
n	3.0	2.8
L_0	100	148
Analysis of $\bar{Y}$ function		
n	3.4	3.5
L_0	72	227

It should be noted that this quantitative analysis of the binding and inhibition data is based on the assumption that $L_0 \geqslant 50$. This is necessary in order to be able to equate $\bar{R}$ with β, and K_T with the K_D at zero bound aurovertin. K_T is necessary to calculate c which is critical for the term $c/(1-c)$ in the plot of the binding data given in Fig. 13. The assumption that $L_0 \geqslant 50$ yields the highest value of c that can be obtained from the Scatchard plot. If the other extreme assumption is made, namely that $c=0$, the binding data yield a value of L_0 of about 10. If this is the case n will become less than 3.4, but still substantially greater than 1. The low value of L calculated at high aurovertin concentrations is independent of the assumption that $L_0 \gg 50$, since, under these conditions the term $c/(1-c)$ is negligible compared with

$$\frac{\bar{Y}}{\alpha-(1+\alpha)\bar{Y}}$$

even when c has its maximum possible value.

This analysis suggests that aurovertin induces a conformation change in the binding site on F_1 in two ways: (1) directly by acting as an allosteric effector of an oligomeric system; (2) indirectly by inhibiting State-3 respiration which changes the allosteric constant of the oligomeric system.

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