THE USE OF THE FLUORESCENT PROBE AUROVERTIN, TO MONITOR ENERGY LINKED CONFORMATIONAL CHANGES IN MITOCHONDRIAL ATPases

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1. Introduction

Structural changes of particular membrane components can be monitored by the use of highly site specific spectroscopic probes, [1–3]. Examples of these are, the use of antimycin not only as an electron transport inhibitor but as a structural probe of the $b-c_1$ segment of the respiratory chain [4]; and of the (Na^+-K^+) ATPase inhibitor strophantidine sulphonaphthylhydrazone as a structural probe for bound ATPases [5]. The fluorescent probe aurovertin [6–8], has been shown to be an inhibitor of oxidative phosphorylation, $^{32}P_i$–ATP exchange, and the exchange of ^{18}O between P_i and H_2O in rat liver mitochondria [9]. In addition aurovertin has been shown to inhibit soluble mitochondrial ATPase [10], and to form stoichiometric complexes with F_1 [11].

This paper sets out to determine the solvent dependent fluorescent characteristics of aurovertin, and to relate these properties to the changes in fluorescence observed on substrate addition when the probe is bound to submitochondrial ATPase. From these findings a model is proposed to explain the inhibitory effect of aurovertin, and its response, in conformational terms, to F_1 substrates.

2. Materials and methods

Fragmented mitochondria were prepared from rat liver mitochondria according to published method [12]. The particles were suspended in a medium containing 0.25 M sucrose, 5 mM Tris–HCl pH 7.5, and 2 mM EDTA. Aurovertin was a gift from Drs. R.B. Beechey and M.D. Osselton (Shell Research, Sittingbourne, Kent). Fluorescence measurements were made using a Hitachi Perkin–Elmer spectrofluorimeter (MPF-2), fitted with temperature regulation. ATPase activity was measured by a standard method [13], NAD reduction by using a Perkin–Elmer 124 spectrophotometer [14]. Fluorescence polarization spectra were measured according to Weber [15].

Abbreviations:
ANS, 8-anilinonaphthalene-1-sulphonic acid; FCCP, carbonyl cyanide *p*-trifluoro methoxyphenylhydrazone.

3. Results

The fluorescence yield of aurovertin is dependent on the viscosity of the medium, but not, unlike ANS, on its polarity. This is clearly illustrated in fig. 1A. The fluorescence is greatly enhanced in the presence of a viscous solvent (glycerol), but very much less so in low polarity solvents (ethanol, methanol and acetone). At 4 °C, the fluorescence quantum yield in glycerol is approximately fourteen times that in ethanol. The effect of reducing the viscosity of the medium by increasing its temperature results in a simultaneous fluorescence decrease. The double reciprocal plot of fluorescence against viscosity is linear (fig. 1B). These results indicate that any changes in fluorescence observed from aurovertin bound to mitochondrial membranes are likely to be the result of changes in the 'viscosity' of the probe's environment, rather than changes in the polarity of the site. This is further borne out by the fluorescence polarization spectra of auro-

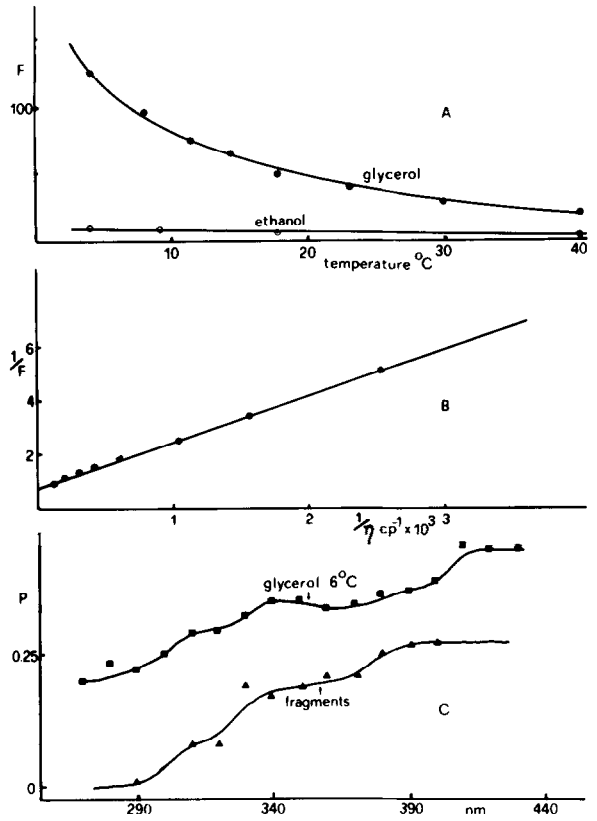

Fig. 1. The effect of the viscosity and of the polarity of the medium on aurovertin fluorescence: A) The fluorescence at 500 nm of 1 μM aurovertin in glycerol or ethanol, measured as a function of temperature (total volume 3 ml); B) The values of the fluorescence from aurovertin in glycerol at different temperatures, replotted in the double reciprocal form of fluorescence against viscosity. The values of the viscosity of glycerol at different temperatures were taken from the Handbook of Chemistry and Physics published by The Chemical Rubber Company 1970; C) The principal polarization spectra of aurovertin in glycerol and bound to membrane fragments. Experimental conditions for the spectrum in glycerol were as in fig. 1A. Membrane fragmets (2 mg/ml) were suspended in: 0.25 M sucrose; 10 mM Tris–HCl, pH 7.5; aurovertin 1 μM, total volume 3 ml. The polariztaion spectra were obtained according to the method of Weber [15].

vertin in glycerol and when bound to membrane fragments (fig. 1C). In glycerol at 6°C, three transitions were resolved, and the maximum polarization (at longer wavelengths), approached the limiting value of 0.5. If the spectrum was measured in water, no polarization of aurovertin fluorescence could be detected. When bound to membrane fragments, intermediate values of polarization were observed (0.25), indicating that the rotational motion of aurovertin was strongly restricted. This aspect will be returned to later.

The emission spectra of aurovertin bound to mitochondrial fragments are shown in fig. 2. An emission maximum was found at 470 nm, accompanied by a shoulder at 490 nm. In fig. 2 A, the addition fo succinate and potassium cyanide can be seen to cause an overall increase (≈ 20%) in the emission spectrum. The addition of ATP, however, results in an overall decrease (≈ 24%) in fluorescence; this effect can be partly or wholly restored by the addition of FCCP (or other uncouplers, not shown).

The difference in fluorescence response caused by the addition of ATP or succinate, can be more clearly seen in fig. 3 A. The effect on the fluorescence of ANS bound to mitochondrial fragments, of the addition of ATP or succinate is also shown to illustrate that aurovertin is obviously not monitoring the same changes as ANS. Fig. 3 B shows the double reciprocal plot of aurovertin fluorescence against protein concentration, in the absence and presence of ATP. The use of such plots to determine quantum yield changes has been strongly challenged [16], but sufficiently high protein concentrations were used for this plot that the objections no longer apply. The results indicate that the addition of ATP causes a 50% reduction of the quantum yield of fluorescence of aurovertin bound to mitochondrial fragments.

The addition of ADP causes an increase in fluorescence (K_m = 75 μM), see fig. 4 A. This increase can be easily abolished by the addition of Mg^{2+} (K_m = 35 μM), fig. 4 B. Other divalent metal cations, such as Ca^{2+} did not seem effective. The Mg^{2+} induced-decrease in fluorescence can in turn be abolished by the addi-

Table 1

	ATP induced fluorescence decrease	ATPase activity* nmoles/ min/mg	NAD reduction* nmoles/ min/mg
0.25 M sucrose	51	18.9	68
1.0 M sucrose	37	13.7	51

* No aurovertin; otherwise conditions as given in fig. 4.

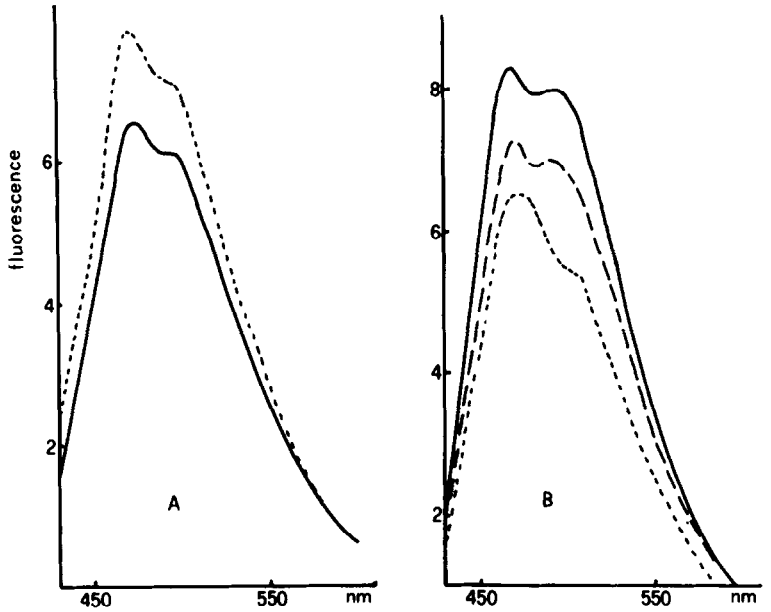

Fig. 2. The emission spectra of aurovertin bound to membrane fragments. Excitation at 360 nm. A) The emission spectra of 1 μM aurovertin bound to membrane fragments (——), and after the addition of 1 mM succinate and 2.5 mM, KCN (– – –). Reaction medium contained: 0.47 mg protein in 0.25 M sucrose; 10 mM Tris–HCl, pH 7.5; 2 mM EDTA, total volume 2 ml; B) The emission spectra of aurovertin bound to membrane fragments (——), after the addition of 1 mM ATP (·····) and after the addition of 0.3 μM FCCP (– – –). Reaction conditions as in fig. 2 A.

tion of EDTA (fig. 4 C), although a considerable amount was required for complete supression of the Mg^{2+} effect (K_m = 500 μM).

The ATP–FCCP induced-fluorescence cycle is inhibited by the presence of oligomycin (fig. 5). The ATP induced-decrease is 90% inhibited by 4 nmoles oligomycin/mg protein, whereas the FCCP induced-reversal of the ATP effect requires much less oligomycin for this amount of inhibition (0.5 nmoles/mg protein).

The viscosity of the suspending medium was increased by increasing its sucrose content from 0.25 M to 1.0 M. The basal aurovertin fluorescence was increased slightly as was expected, more interesting however was the effect on the ATP induced decrease in fluorescence. The increase in the viscosity of the medium resulted in a marked decrease in the effectiveness of ATP to reduce the aurovertin fluorescence. This decrease in effectiveness was found to correlate exactly with the decrease in the efficiency of two energy linked functions (see table 1). This increase in the viscosity of the medium resulted in a decrease in the effectiveness of each of these processes by 26%. It was also found that if the medium was made very viscous (≈ 2 M sucrose), that ATP was unable to cause any decrease in aurovertin fluorescence, that there was no ATPase activity and that reversed electron transport was prevented.

4. Discussion

An initial conclusion of this note is that aurovertin is able to monitor some different property of mitochondrial membranes to that monitored by ANS. ANS, which is probably delocalized over the entire membrane (n = 40–50 nmoles/mg protein [17]), can be used as a general probe of membrane structure, whilst aurovertin (n = 0.2 nmoles/mg protein [18] can only probe localized structural changes. The fluorescence changes of aurovertin when bound to mitochondrial membranes may be interpreted in terms of environmental constraint of the probe binding site. In fact the fluorescence is enhanced specifically in highly viscous media.

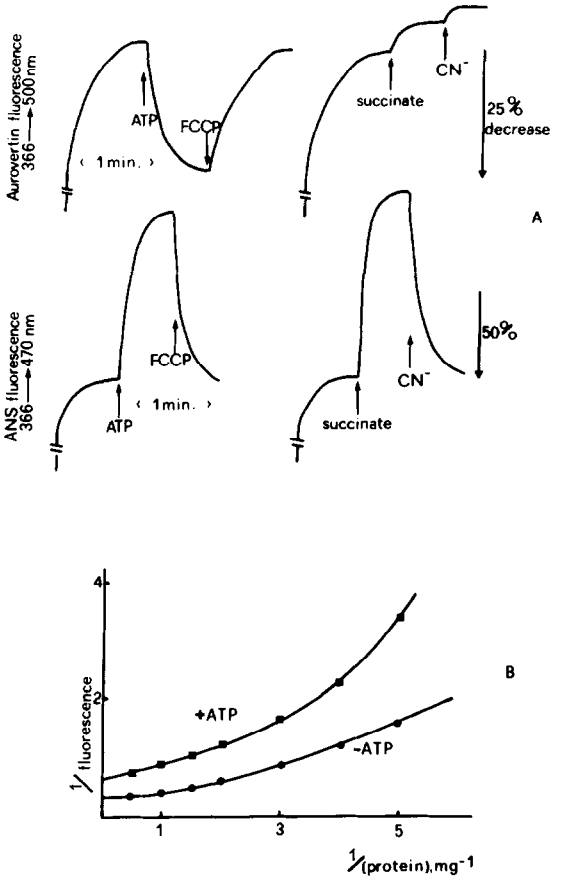

Fig. 3. A) Energy linked changes in the fluorescence from aurovertin and ANS bound to membrane fragments. Reaction medium contained: 0.47 mg protein in 0.25 M sucrose; 10 mM Tris–HCl, pH 7.5; 2 mM EDTA, total volume 3 ml. Aurovertin concentration 2 μM, ANS 12 μM. Additions were 1 mM ATP, 1.5 mM succinate, 2.5 mM KCN. B) The double reciprocal plot of aurovertin fluorescence against protein concentration in the presence and absence of 1 mM ATP. Medium and aurovertin concentration as given in fig. 3 A.

While changes in binding of ATP and ADP might affect the conformation of the ATPase complex, the aurovertin fluorescence changes produced by ATP are most probably related to changes in the energy state of the membrane. In fact, in the presence of excess ATP, uncouplers are still able to reverse the fluorescence change produced by ATP (fig. 3). The effect of oligomycin, which does not prevent ATP binding to the complex, but does inhibit the ATP induced decrease in aurovertin fluorescence is in favour of the above interpretation. The fluorescence increase obtained in the presence of ADP may be the simple consequence of the binding of the nucleotide to its site.

Alternatively, reversible binding of F_1 inhibitor [19] to the ATPase complex may be relevant to the interpretation of the fluorescence changes induced by ADP, Mg^{2+} and ATP.

The titration of the ATP induced aurovertin fluorescence change and its reverse, with oligomycin, suggests that ATP produces a structural change in the ATPase complex which is only sensitive to high concentrations of oligomycin. The oligomycin sensitive step of oxidative phosphorylation may be synonomous with the reversal of the ATP induced-fluorescence decrease, both occurring at low oligomycin concentrations (0.2–0.5 nmoles/mg protein).

The results presented here may be explained in terms of a simple constraint switch mechanism as follows: ATP causes a binding of the ATPase inhibitor, resulting in a decreased constraint of the aurovertin binding site, and low fluorescence. The presence of ADP however induces the release of the inhibitor, and a consequential increased constraint of the aurovertin site and an increase in the fluorescence. The effects of ATP and ADP on inhibitor binding then enable the possible regulatory nature of these substrates on oxidative phosphorylation to be accounted for.

In addition, Mg^{2+} and EDTA have been reported [20] to cause the binding and the release of the ATPase inhibitor respectively. Their effect on the fluorescence from aurovertin bound to mitochondrial fragments, can equally be explained in terms of the proposed constraint switch mechanism.

The suggestion that constraint may be an important parameter in energy conserving mechanisms, is supported by the observations of the effect of increasing the viscosity of the medium. High viscosity not only seemed to prevent to some extent the operation of the constraint switch by ATP, but also reduced the rates of some energy-linked functions (see table 1).

While this paper was in preparation, a paper by Chang and Penefsky was published [21] where effects on aurovertin fluorescence produced by ATP, ADP and Mg^{2+} and respiratory substrates in mitochondrial fragments were also seen. Some differences, namely, the lack of energy dependent responses, the effect with succinate as a substrate in our conditions; may be due to the different kind of membrane fragments utilized

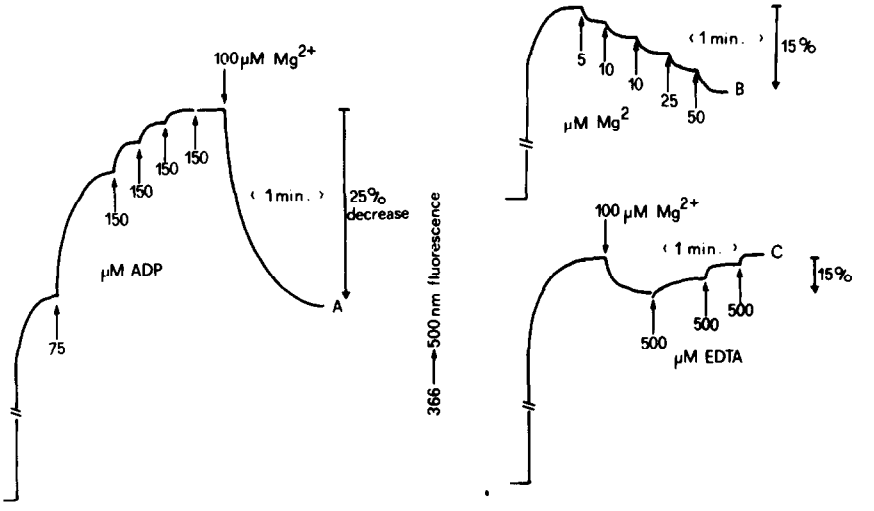

Fig. 4. A) Titration of the effect of ADP on aurovertin fluorescence; B) Titration of the effect of Mg^{2+} on aurovertin fluorescence; C) Titration of the reversal of the Mg^{2+} reduction of aurovertin fluorescence by EDTA. Reaction conditions as given in fig. 3 A.

by Chang and Penefsky in their experiments. Similarly, the lack of energy dependent changes, induced by ATP and reversed by FCCP may be the consequence of the higher content of F_i inhibitor in ETPH (used by Chang and Penefsky) with respect to the EDTA fragments used in this study.

In conclusion, the use of highly, site-specific probes such as aurovertin, appears to have great potential in the study of the structure–function relationships of bound ATPases.

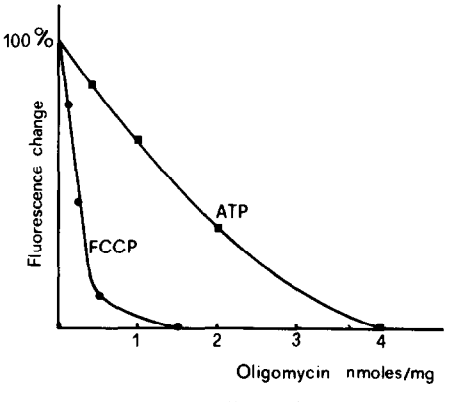

Fig. 5. The titration of the inhibition by oligomycin of the changes in aurovertin fluorescence produced by ATP and FCCP. Conditions as given in fig. 3 A, ATP concentration 1 mM, FCCP concentration 0.3 μM.

Acknowledgements

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