THE EFFECT OF SOME UNCOUPLING AGENTS, IONOPHOROUS AGENTS AND INHIBITORS ON THE FLUORESCENCE OF ANS BOUND TO BOVINE SERUM ALBUMIN

Derek LAYTON and Peter SYMMONS

Biophysics Laboratory, Chelsea College, Manresa Road, London, SW3 6LX, England

Received 10 January 1973

1. Introduction

The energization of sub-mitochondrial particles is known to cause an increase in ANS fluorescence [1]; this increase is generally thought to be due, in part at least, to an increase in the amount of dye bound to the membrane. The addition of uncouplers causes the fluorescence to fall to the pre-energized level [1]. It has been suggested that the rapid interaction of ANS with bovine serum albumin may afford the direct measurement of free ANS [2] in such systems.

Bovine serum albumin (BSA) is able to bind up to five molecules of ANS [3]. By using sufficient BSA in the external medium such that its sites are never fully occupied, BSA can be used to monitor the amount of free ANS in the external medium. The proportions of BSA, ANS and sub-mitochondrial particles, can be made such that the ANS–BSA complex fluorescence "swamps" the ANS-membrane fluorescence. Movement of the dye onto the membrane on energization will decrease the free dye in the medium and result in a decreased equilibrium for ANS bound to BSA, the opposite occurring on uncoupling and the ANS–BSA complex fluorescence will change accordingly.

Similarly, particles can be made with sufficient BSA trapped inside the particle, such that the electrophoretic movement of the dye on energisation [4] can be verified by observations of the changes in ANS–BSA fluorescence.

During a series of controls carried out to determine whether the mitochondrial membrane was permeable to BSA, or whether BSA bound to the membrane, some effects were observed that led to the experiments reported here.

2. Materials and methods

The magnesium salt of ANS, obtained from Eastman Kodak Ltd., was recrystallized twice from hot solutions. BSA and oligomycin were obtained from Sigma Chemical Corp., and carbonyl cyanide m-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) from Dupont. Valinomycin, nigericin, gramicidin and tetrachlorotrifluoromethyl benzimidazole (TTFB) were gifts from Professor H. Baum. The *N*-methyl derivative of TTFB was synthesized for us by Professor R.B. Beechy of Shell Research Limited, Sittingbourne, Kent, to whom we are indebted. All other chemicals were Analar grade. Fluorescence measurements were made using a spectrofluorimeter designed and constructed in this laboratory.

3. Results and discussion

It was found using ANS to monitor the energized state of submitochondrial particles with sufficient BSA in the external medium to meet previously described requirements, that uncoupling of the membrane by FCCP, (i.e. a release of ANS into the external medium) caused a decrease rather than the expected increase in ANS–BSA fluorescence. When potassium cyanide was used instead of FCCP, the expected increase in fluorescence was observed. In the absence of any particles, FCCP still reduced the ANS–BSA fluorescence, whereas cyanide had no effect. Using the detergent Triton X-100, the enhanced ANS fluorescence was also markedly reduced by the intro-

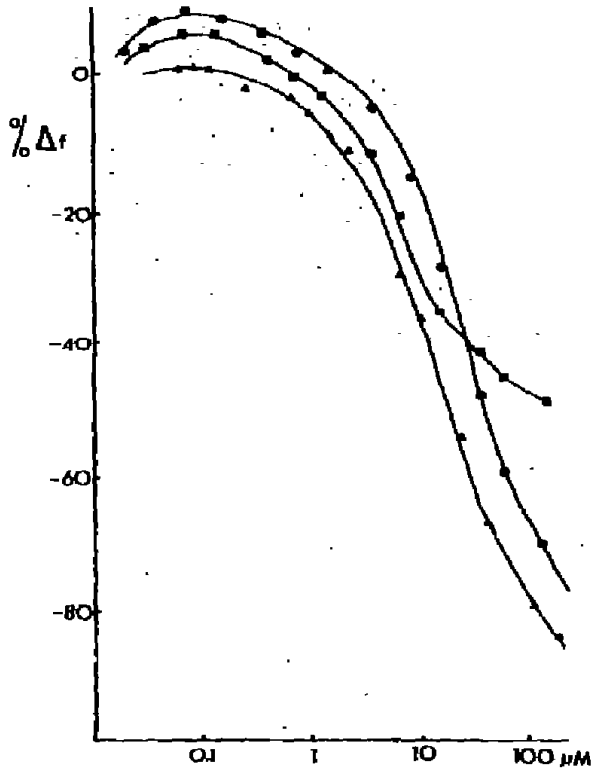

Fig. 1. Percentage change in enhanced fluorescence from 5×10^{-6} M ANS in 0.8 mg/ml BSA, total volume 3 ml. (●—●—●) FCCP; (■—■—■) TTFB; (▲—▲—▲) CCCP.

duction of FCCP but not by cyanide. This reduction of the enhanced fluorescence could be due to displacement of ANS by FCCP; disturbance of the ANS site, possibly by affecting an ordered water layer, or by

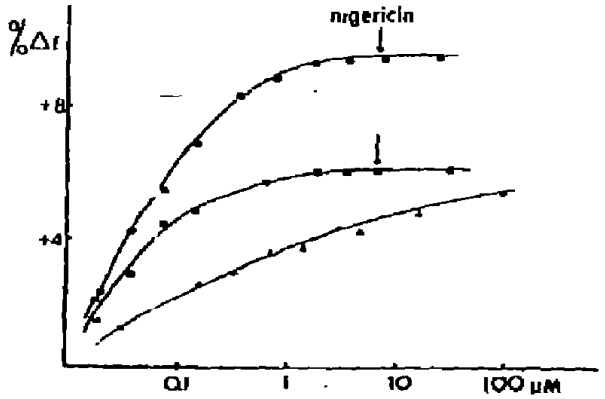

Fig. 2. Percentage change in enhanced fluorescence from 5×10^{-6} M ANS in 0.8 mg/ml BSA, total volume 3 ml. (●—●—●) Valinomycin in 10 mM KCl; (■—■—■) valinomycin; (▲—▲—▲) oligomycin. Nigericin addition = 10 μM.

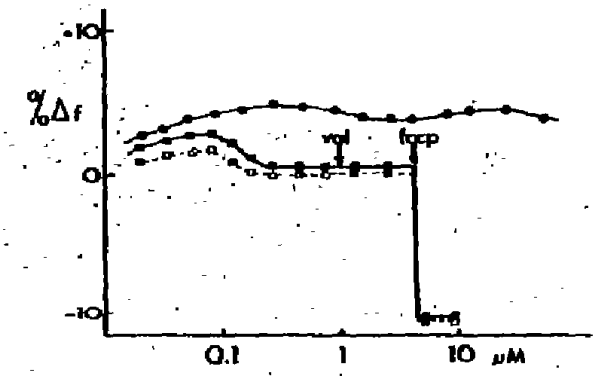

Fig. 3. Percentage change in enhanced fluorescence from 5×10^{-6} M ANS in 0.8 mg/ml BSA, total volume 3 ml. (●—●—●) Gramicidin; (■—■—■) nigericin in 10 mM KCl; (□—□—□) nigericin. Valinomycin addition = 1 μM; FCCP addition 6.67 μM.

energy transfer to FCCP or its complex. In the solvent dioxane, the high fluorescence yield was only slightly reduced, even at high FCCP concentrations (> 100 μM). The large decrease in fluorescence yield caused by the addition of FCCP, was only seen when ANS was bound to BSA, detergent micelles etc., in which the dye was in a localized hydrophobic environment, but within an aqueous system.

The titrations of the percentage change in fluorescence yield with the uncouplers FCCP, CCCP and TTFB are shown in fig. 1; the change in fluorescence

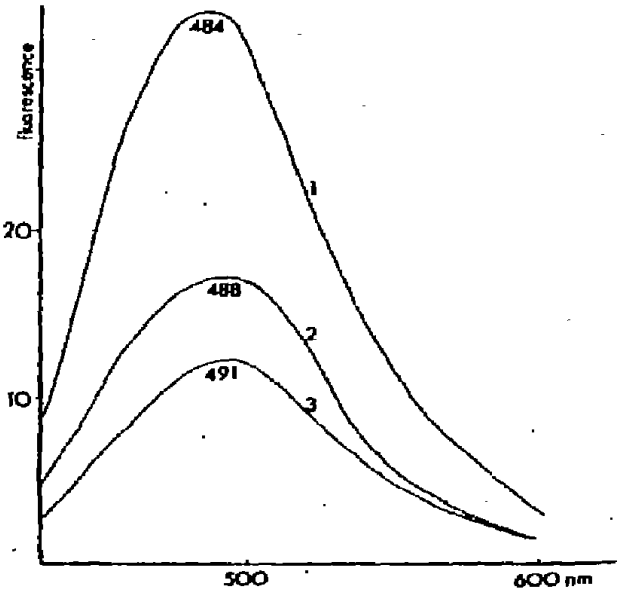

Fig. 4. Emission spectrum of 5×10^{-6} M ANS in 0.8 mg/ml BSA, total volume 3 ml. 1) No FCCP; 2) 13 μM FCCP; 3) 20 μM FCCP.

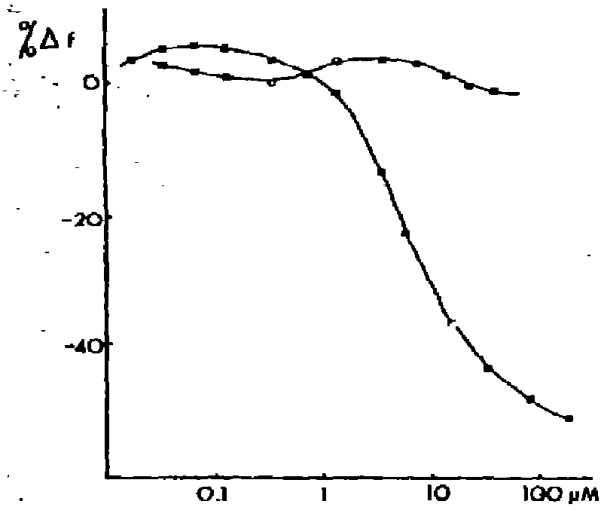

Fig. 5. Percentage change in enhanced fluorescence from 5×10^{-6} M ANS in 0.8 mg/ml BSA, total volume 3 ml. (●—●—●) TTFB; (□—□—□) *N*-methyl TTFB.

having been corrected for the small changes due to the introduction of ethanol (the uncoupler solvent) into the system. Over the range 1 μM–100 μM, there is considerable reduction of fluorescence yield, by all three uncouplers; TTFB exhibits saturation, whereas FCCP and CCCP are capable of eliminating all the BSA enhancement of ANS fluorescence.

The ionophorous agents, or K^+ dependent uncouplers, produce a similar initial rise to FCCP etc., but this increased enhancement is maintained and plateaus, no reduction of fluorescence is observed (figs. 2 and 3). The addition of nigericin to valinomycin or vice versa, had no effect on the fluorescence from this system. As expected, the effect seen for these agents is not markedly affected by the presence or absence of 10 mM KCl. Gramicidin shows a slight enhancement of fluorescence, as does the ATP-ase inhibitor oligomycin, although higher concentrations of the latter are required to reach its plateau.

The emission spectra of ANS–BSA in the absence and presence of FCCP are shown in fig. 4. The reduction of the enhanced fluorescence with increasing uncoupler concentration is again apparent, as is the shift in the emission maxima to the red. The shift from 484–491 nm corresponds to a change in Z value [5] from 76–82. It would appear that the effect is partly due to a change in the environment of the probe; and partly due to a decrease in the amount of probe bound, either indirectly by a change in binding constants, or as a result of competition of FCCP for the same site, or some combination of both. The effect of the ionophorous agents and the inhibitor oligomycin in producing some small constant increase in enhancement of fluorescence, may be the result of an increase in the hydrophobicity of the site, or an increase in the amount of probe bound. The increase would correspond to a shift in emission maxima of less than 1 nm, of which reliable measurement was not possible.

The uncharged derivative of TTFB, i.e., *N*-methyl TTFB, which is not thought to be an uncoupler, did not produce a decrease in the enhanced fluorescence when titrated similarly, (fig. 5). At very high concentrations (> 200 μM) there was some decrease in fluorescence enhancement, and an accompanying shift in emission maxima; but this may be due to residual traces of TTFB. As the only structural difference between the effective uncoupler TTFB and the non effective *N*-methyl derivative is one of charge, the mode of action of such uncouplers may be charge dependent, and this may account for the effectiveness of TTFB on BSA mediated fluorescence.

The results presented here, show that for the protein BSA; FFCP, CCCP and TTFB interact in such a way as to affect the ANS binding site and to decrease the amount of probe bound. These uncouplers also produce similar effects on the fluorescence of ANS with detergent micelles, and the assumption may be made that they will produce similar effects on the fluorescence of ANS when bound to mitochondrial membranes. Both the substituted benzimidazole and carbonyl cyanide phenylhydrazone uncouplers are 100% effective at substoichiometric concentrations and have been shown [6] to act preferentially on "active phosphorylation sites". It is of interest that ANS also has an uncoupling action [7].

Although it is accepted that changes in ANS fluorescence on energization of mitochondrial systems are the result mainly of changes in the amount of probe bound, with some alteration of quantum yield, there has not to date been much information as to nature and position of the ANS binding site for such systems other than that it is hydrophobic.

Whilst it would seem most likely that the changes in ANS fluorescence observed on uncoupling result independently and directly from the action of the uncoupler, the possibility that the uncoupers we have

examined interact directly with the ANS binding site is an inference from our observations. It may be that both ANS and these uncouplers bind to similar regions of the mitochondrial membrane closely associated with the coupling reaction, and that this site is a hydrophobic region of a protein.

However, these results appear to indicate that in using ANS fluorescence as an "indicator" of the energy state of the mitochondrial system, the effects of some uncouplers on the fluorescence should be treated with caution before being ascribed directly to reflect the state of the membrane.

References

[1] A. Azzi, B. Chance, G.K. Radda and C.P. Lee, Proc. Natl. Acad. Sci. U.S. 62 (1969) 612.
[2] A. Azzi, P.L. Gherardini and M. Santato, in: Energy Transduction in Respiration and Photosynthesis, eds. E. Quagliariello, S. Pappa and C.S. Rossi (Adriatica Editrice Bari, 1971) p. 621.
[3] E. Daniel and G. Weber, Biochemistry 5 (1966) 1893.
[4] A.A. Jasaitis, V.V. Kuleinne and V.P. Skulachev, Biochim. Biophys. Acta 234 (1971) 177.
[5] E.M. Kosower, J. Am. Chem. Soc. 80 (1958) 3253.
[6] S.A. Margolis, G. Lenaz and H. Baum, ARch. Biochem. Biophys. 118 (1967) 224.
[7] D. Layton, P. Symmons and P. Williams, Biochem. Soc. Trans. 1 (1972) 92, in press.