Biochimica et Biophysica Acta, 339 (1974) 157–163

BBA 76582

THE INFLUENCE OF DIFFUSION POTENTIALS ACROSS LIPOSOMAL MEMBRANES ON THE FLUORESCENCE INTENSITY OF 1-ANILINO-NAPHTHALENE-8-SULPHONATE

EVERT P. BAKKER and KAREL VAN DAM

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

(Received September 25th, 1973)

SUMMARY

The influence of diffusion potentials across different phospholipid membranes on the fluorescence intensity of 1-anilinonaphthalene-8-sulphonate (ANS) was studied. With liposomes or chloroform spheres covered with a monolayer of egg lecithin, no specific effects were found. With liposomes of soy-bean phospholipids, generation of a diffusion potential leads to an enhancement or decrease, depending on the direction of the potential, of the intensity of ANS fluorescence. This effect is mainly due to a change in quantum yield of the bound ANS. These data support a mechanism according to which ANS molecules are pushed into or pulled out of the membrane by a potential, but not an electrophoretic one in which the potential causes movement of ANS across the membrane.

INTRODUCTION

The fluorescence intensity of the probe 1-anilinonaphthalene-8-sulphonate (ANS) is quenched upon energization of intact mitochondria [1–4]. With fragmented mitochondria, in which the orientation of the membrane is inside-out [5], however, an enhancement of the fluorescence intensity of the fluorochrome is observed upon energization [1–4, 6–9]. As these effects can be induced by energization with either succinate or ATP [1, 2], as well as by diffusion potentials generated across the membrane of the energy-conserving organelle [3, 4, 6, 8], the question arises whether those effects of ANS are caused by conformational changes of the protein moiety of the membrane or by direct effects of the potential across the organelle membrane on the ANS molecule. It is difficult to decide between these alternatives, since the high-energy conformation and the potential across the membrane of the energy-conserving membrane are in equilibrium [10].

Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonate; 1799, α,α′-bis(hexafluoroacetonyl)-acetone.

Patrick et al. [11] report a direct relationship between the potential across the electroplax of *Electrophorus electricus* and the fluorescence intensity of ANS. These authors could not decide between a mechanism of changed quantum yield of ANS bound to the membrane and differences in the amount of bound ANS, due to conformational changes in the membrane, but reject an electrophoretic mechanism in which ANS is transported across the membrane [11], as has been proposed by Jasaitis et al. [3] for mitochondria and submitochondrial particles.

In this paper we report the results of experiments with liposomes, in which we generated diffusion potentials across the membrane, in order to eliminate the effects that conformational changes of membrane proteins may have on the fluorescence intensity of ANS. Such diffusion potentials generated across the membrane of liposomes prepared from soy-bean phospholipids lead to effects on the fluorescence intensity of ANS similar to those observed with mitochondria and submitochondrial particles.

MATERIALS AND METHODS

Egg lecithin was prepared according to the method of Pangborn [12].

Soy-bean phospholipids were purified from asolectin according to the method of Kagawa and Racker [13].

Liposomes were prepared as described previously [14]. Briefly, 100 mg phospholipid was shaken with glass beads in 10 ml of a solution containing 1 mM EDTA and 25 mM Tris–HCl (pH 7.5) or 25 mM KCl plus 5 mM Tris–HCl (pH 7.5). After sonication under argon atmosphere until the solution was visually clear, the medium was, if necessary, exchanged for one containing another monovalent chloride salt (25 mM) by chromatography on a Sephadex G-75 column.

Monolayer vesicles, 550-Å droplets of chloroform in water, covered with a monolayer of phospholipid, were prepared according to the method of Träuble and Grell [15].

An Eppendorf 1101 M fluorimeter was used to measure the fluorescence intensity of ANS. Filters of 313 plus 366 nm and 470–3000 nm were used for excitation and emission light, respectively.

Twice recrystallized ANS was a gift of Dr G. K. Radda, Department of Biochemistry, University of Oxford, Oxford, Great Britain. Valinomycin, nigericin and monensin were gifts of Dr W. C. Pettinga, Eli Lilly and Comp., Indianapolis, U.S.A.

RESULTS

Egg-lecithin monolayer vesicles

The time course of the change in fluorescence intensity of ANS, added to egg-lecithin monolayer vesicles suspended in different monovalent salt media, is always biphasic. The slow phase lasts approx. 1 h after, which a stable level is reached (Fig. 1A). Addition of ionophores results in an increase of the rate of the slow phase by one or two orders of magnitude (Fig. 1B). However, this effect is not specific for conditions under which a diffusion potential is generated across the membrane and seems to represent the complexing of the cations with the ionophore in the membrane region [16, 17], since in a NaCl medium plus valinomycin or monensin (not shown)

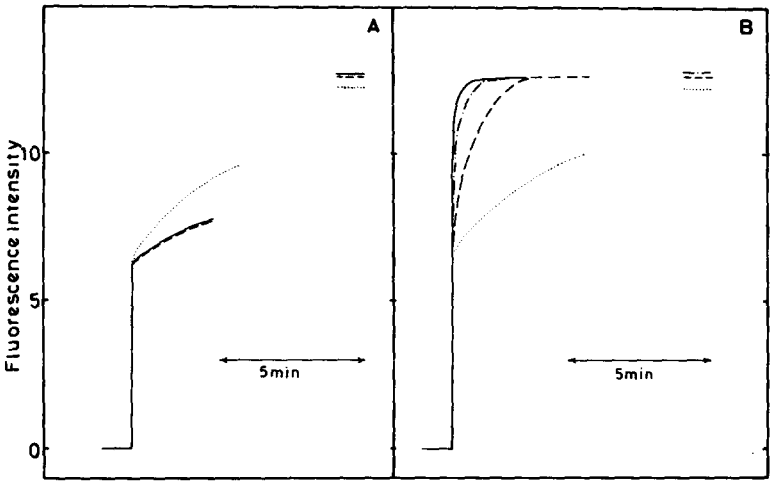

Fig. 1. The time course of the fluorescence of ANS added to monolayer vesicles covered with lecithin. Monolayer vesicles (0.4 mg lecithin) were suspended in 25 mM monovalent chloride salts. At $t = 0$ ANS (10 μM) was added. The time course of the fluorescence was monitored. (A) Without ionophores. · · ·, 25 mM Tris–HCl (pH 7.5); —, 25 mM KCl; - - -, 25 mM NaCl. (B) In the presence of ionophores (500 ng). —, 25 mM KCl plus valinomycin; - - -, 25 mM NaCl plus valinomycin; - · - · -, 25 mM KCl plus nigericin; . . ., 25 mM Tris–HCl (pH 7.5) plus valinomycin.

and a KCl medium plus nigericin effects similar to that in a KCl medium plus valinomycin are observed (Fig. 1B). Essentially the same results were obtained with sonicated liposomes prepared from egg lecithin.

Soy-bean phospholipids

The fluorescence intensity of ANS in the presence of liposomes or monolayer vesicles prepared from soy-bean phospholipids is one fifth of that of ANS in the presence of egg-lecithin vesicles. This effect is caused by the net negative surface charge of the former liposomes [18].

As in the experiments with egg-lecithin vesicles, the time course of the fluorescence of ANS added to liposomes prepared from soy-bean phospholipids is biphasic (Fig. 2A). Generation of a diffusion potential positive inside and negative outside the vesicle by adding KCl plus valinomycin to liposomes prepared in Tris–HCl now specifically causes a large transient increase in the fluorescence intensity of ANS (Fig. 2B). Note, that the effect, and the sign of the potential are the same as observed in submitochondrial particles. In a NaCl medium plus valinomycin or in a KCl medium plus nigericin the rate of the slow phase of the fluorescence increases (Fig. 2B), similar to the situation with egg-lecithin vesicles, probably as a consequence of the complexing reaction of the ionophores with the monovalent cations present [16]. The same results were obtained with monolayer vesicles covered with soy-bean phospholipids.

In Fig. 3 a double reciprocal plot is given of the fluorescence of ANS shortly after addition of valinomycin and after complete equilibration in a KCl medium as a function of the amount of liposomes. Extrapolation to infinite liposome concentration indicates that the transient enhancement observed under these conditions is largely

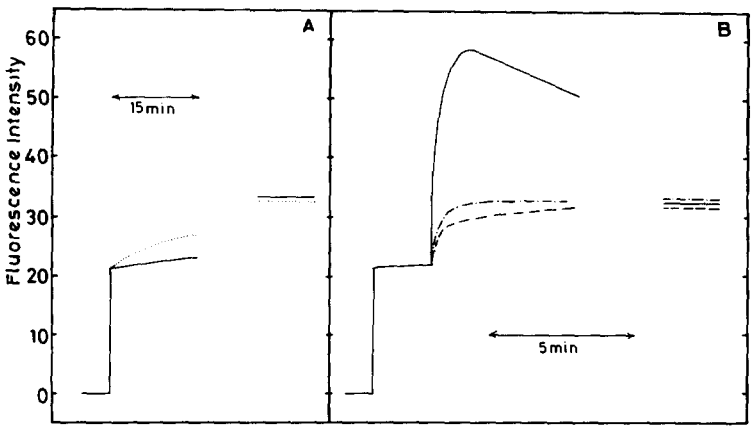

Fig. 2. The time course of the fluorescence intensity of ANS added to liposomes prepared from soy-bean phospholipids. Liposomes (2.5 mg phospholipid) prepared in 25 mM Tris–HCl (pH 7.5) were suspended in 25 mM monovalent chloride salts. At $t = 0$ ANS (10 μM) was added. The time course of the fluorescence was monitored. Symbols: the same as in Fig. 1. (A) Without antibiotic. (B) 250 ng antibiotic were added at the time of the second increase in fluorescence.

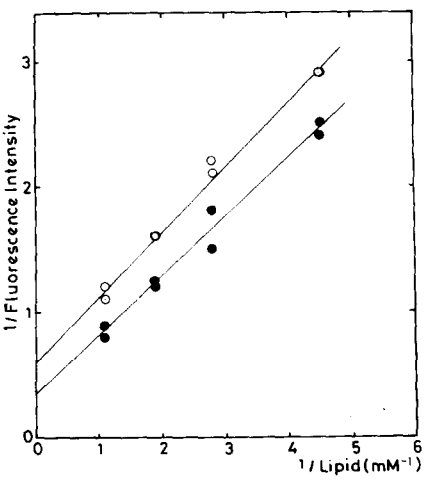

Fig. 3. A double reciprocal plot of the fluorescence intensity of ANS as a function of the soy-bean phospholipid concentration, after addition of valinomycin in a KCl medium. The conditions were as described in the legend to Fig. 2. The ratio phospholipid/valinomycin was constant (1 mole of valinomycin per 3000 moles of lipid). ●–●, the reciprocal of the maximum fluorescence level after addition of valinomycin; ○–○, the reciprocal of the final fluorescence level.

the result of a change in the quantum yield of the fluorescence of ANS. This result is different from the one obtained upon energization of submitochondrial particles with salt fluxes, where the enhancement in fluorescence of ANS almost completely is caused by an enhanced binding of the probe (not shown).

In Fig. 4 an experiment is depicted in which potentials across the liposomal membrane, opposite in sign to the one shown in Figs 1 and 2, lead to a quenching of the fluorescence intensity of ANS. Such potentials, negative inside the vesicle mem-

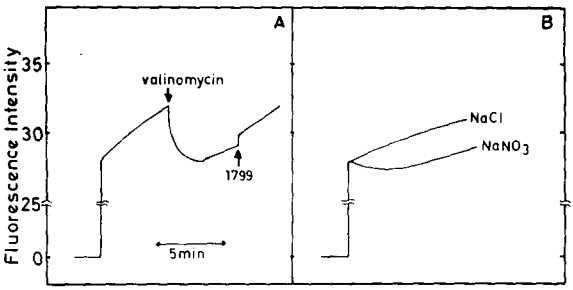

Fig. 4. The time course of the fluorescence intensity of ANS added to liposomes prepared from soy-bean phospholipid loaded with KCl. Liposomes from soy-bean phospholipids were prepared in 25 mM KCl, 5 mM Tris–HCl (pH 7.5). The KCl outside the vesicles was exchanged for 25 mM Tris–HCl (pH 7.5) on a Sephadex G-75 column. These KCl-loaded liposomes (2 mg) were suspended in different monovalent salts. At $t = 0$ ANS (10 μM) was added. (A) The liposomes were suspended in 25 mM Tris–HCl (pH 7.5). Valinomycin (250 ng) and 1799 (10 μM) were added. (B) The liposomes were suspended in 25 mM $NaNO_3$ or 25 mM NaCl.

brane and positive outside can be generated by outflow of K^+, mediated by valinomycin (Fig. 4A) or inflow of NO_3^- (Fig. 4B). Efflux of K^+, and also influx of NO_3^- (if compared with Cl^-), caused quenching of the fluorescence. The uncoupler 1799 reversed the quenching, but not completely (Fig. 4A). This is probably due to a direct effect of the uncoupler on the interaction between lipid and ANS, leading to a lower fluorescence of the latter molecule [19].

In Fig. 5 an experiment is shown where the KCl concentration in a medium containing liposomes loaded with 25 mM KCl was varied. Up to a concentration of 3 mM KCl outside the vesicle, a quenching of the fluorescence intensity of ANS is observed after addition of valinomycin. At higher concentrations of KCl in the medium an enhancement occurs, representing the complexing of valinomycin with K^+

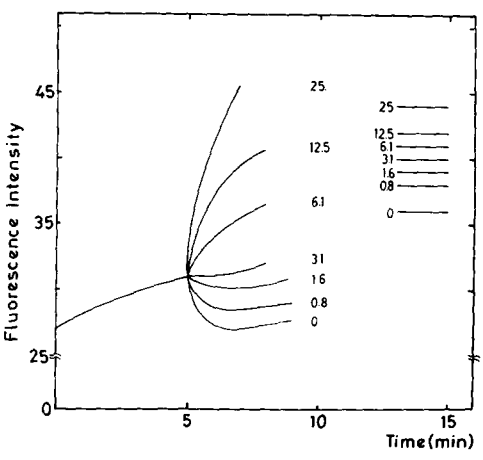

Fig. 5. The time course of the fluorescence intensity of ANS added to liposomes prepared from soy-bean phospholipids loaded with KCl and suspended in media containing different KCl concentrations. The conditions were as described in the legend to Fig. 4. The concentration of KCl in the medium was as indicated in the figure. In addition Tris–HCl (pH 7.5) was added, so that the concentration of KCl plus Tris–HCl in the medium was 25 mM. At $t = 5$ min 250 ng valinomycin was added.

described above [16]. The mutual interference of the complexing and the potential effects makes it difficult to obtain quantitative data of the potential across the liposomes from measurements of the fluorescence intensity of ANS.

DISCUSSION

The observation that diffusion potentials across membranes of liposomes bearing a net negative surface charge cause changes in the fluorescence intensity of the probe ANS similar to those observed in submitochondrial particles and intact mitochondria is important because no proteins are involved in the liposomal system, and the observed effects with these particles can only be caused by the membrane potential itself. Therefore, at least some effects observed with mitochondria, submitochondrial particles or the electroplax of *E. electricus* are probably caused directly by the potential across the membrane, and not by conformational changes of the protein moiety of the membrane. In this respect a recent report of Brocklehurst et al. [20] is interesting. These authors failed to observe an energy-dependent change of the fluorescence intensity of the ANS analogue 2-toluidinylnaphthalenesulphonyl chloride, covalently bound to the protein moiety of submitochondrial particles [20]. They conclude that "probe movement is essential for any energy-dependent response" [20].

However, it should be pointed out, that the changes of the fluorescence intensity of ANS reported here, seem to be mainly changes in the quantum yield of the probe molecule bound to the liposome (Fig. 3), in contrast to submitochondrial particles, in which case the changes are changes in the quantum yield plus changes in binding or even changes in binding of ANS alone. Therefore, our results, as well as the results of Patrick et al. [11], can not be used to support the electrophoretic mechanism of ANS across the membrane as proposed by Jasaitis et al. [3]. Our results with liposomes indicate a pushing into or a pulling out of the membrane of the ANS molecule, depending on the sign of the potential across the membrane.

NOTE ADDED IN PROOF (received January 24th, 1974)

After finishing this manuscript our attention was drawn to the work of Conti and Malerba [21]. These authors measured the fluorescence intensity of ANS added to bilayers formed from soy-bean phospholipids in decane as a function of voltage pulses across the bilayer. From -150 to $+150$ mV a proportionality was observed between the voltage applied and the percentage change in fluorescence, if ANS was added to one side of the membrane. No effects were observed after addition to both sides. Our results are in accordance with this report, if the difference in internal and external volume of the liposomes is taken into account.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 Azzi, A. (1969) Biochem. Biophys. Res. Commun. 37, 254–260
2 Azzi, A., Chance, B., Radda, G. K. and Lee, C. P. (1969) Proc. Natl. Acad. Sci. U.S. 62, 612–619
3 Jasaitis, A. A., Kuliene, V. V. and Skulachev, V. P. (1971) Biochim. Biophys. Acta 234, 177–181
4 Haaker, H., Berden, J. A., Kraayenhof, R., Katan, M. and Van Dam, K. (1972) in Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G. F., Carafoli, E., Lehninger, L. A., Quagliariello, E. and Siliprandi, N., eds), pp. 329–339, Academic Press, New York
5 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
6 Brocklehurst, J. R., Freedman, R. B., Hancock, D. J. and Radda, G. K. (1970) Biochem. J. 116, 721–731
7 Azzi, A., Gherardini, P. and Santato, M. (1971) J. Biol. Chem. 246, 2035–2042
8 Radda, G. K. (1971) Biochem. J. 122, 385–396
9 Barrett-Bee, K. and Radda, G. K. (1972) Biochim. Biophys. Acta 267, 211–215
10 Slater, E. C. (1967) Eur. J. Biochem. 1, 317–326
11 Patrick, J., Valeur, B., Monnerie, L. and Changeux, J. P. (1971) J. Membrane Biol. 5, 102–120
12 Pangborn, M. C. (1951) J. Biol. Chem. 188, 471–476
13 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
14 Bakker, E. P., Van den Heuvel, E. J., Wiechmann, A. H. C. A. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 78–87
15 Träuble, H. and Grell, E. (1971) Neurosci. Res. Prog. Bull. 9, 373–386
16 Haynes, D. H. (1972) Biochim. Biophys. Acta 255, 406–410
17 McLaughlin, S. G. A., Szabo, G. and Eisenman, G. (1971) J. Gen. Physiol. 58, 667–687
18 Vanderkooi, J. and Martonosi, A. (1969) Arch. Biochem. Biophys. 133, 153–163
19 Layton, D. and Symmons, P. (1973) FEBS Lett. 30, 325–328
20 Brocklehurst, J. R., Cierkosz, B. and Lee, C. P. (1973) Abstr. 9th Int. Congr. Biochem., Stockholm, p. 235, Aktiebolaget. Egnellska, Boktryckeriet, Stockholm
21 Conti, F. and Malerba, F. (1972) Biophysik 8, 326–332