SPECTROSCOPIC RESOLUTION OF DRUG BINDING SITES IN BIOLOGICAL MEMBRANES

A. G. LEE, Jane ROGERS, D. C. WILTON, K. P. GHIGGINO+ and D. PHILLIPS+
Department of Biochemistry and +*Department of Chemistry, University of Southampton, Southampton, SO9 3TU, England*

Received 3 August 1978

1. Introduction

Spectroscopic techniques have been widely used to obtain information about simple lipid bilayers and purified lipid–protein complexes. The same techniques have also been used to study intact biological membranes but the results of such studies have been much more difficult to interpret, simply because the membrane is a highly heterogeneous structure. In general a single composite spectrum is obtained either from all components of the membrane or from probe molecules dissolved in a variety of sites in the membrane. We now show that the technique of nanosecond time-resolved fluorescence spectroscopy can be used to resolve spectra for fluorescence probes dissolved in different environments within biological membranes.

2. Materials and methods

8-Anilino-1-naphthalenesulphonate (ANS) was obtained as the magnesium salt from Eastman Kodak Co. and twice recrystallised. The microsomal membrane fraction from *Tetrahymena pyriformis* was prepared as in [1]. Steady state fluorescence measurements were made on an Aminco-Bowman SPF fluorimeter. Time-resolved fluorescence measurements were made on an instrument, detailed in [2], which uses the frequency doubled output of a mode-locked cavity-dumpted argon-ion laser as an excitation source (λ_{exc} 275.25), single photon counting detection and some novel gating electronics. Features of this system are the very high laser repetition rate (5 MHz) and intensity which allow the rapid recording of fluorescence lifetimes and time-resolved emission spectra and the exact reproducibility of pulse shape enabling accurate computer analysis of the fluorescence decay kinetics. For the present work a fluorescence spectral resolution of 5 Å was used.

3. Results and discussion

ANS has been much used as a fluorescence probe since it exhibits a large increase in fluorescence intensity and spectra shifts when bound to certain proteins [3], lipids [4] or cellular membranes [4,5]. However, it is uncertain to which components of cellular membranes the ANS binds. Although ANS can bind to protein solubilized from some membranes [6], it is generally thought that the major binding sites are lipid in nature. Thus although proteolysis of intact myelin or erythrocyte membranes has little effect on ANS binding, phospholipase treatment has a large effect [5]. On the other hand, the fluorescence decay for ANS bound to red blood cells has been fitted [7,8] to a double exponential, one component of which is characteristic for the fluorescence decay of ANS bound to lipid and the other for ANS bound to a protein such as apomyoglobin.

ANS is suitable for our studies for two reasons:

(1) ANS bound to either lipid or protein (bovine serum albumin, BSA), has no solvent-dependent spectral shifts on the nanosecond time scale, unlike other probes such as 2-*p*-toluidinylnaphthalene-6-sulphonate [9,10].

(2) There is an easily detectable difference in fluorescence emission maxima for ANS bound to lipid and bound to BSA (fig.1).

Fluorescence decays for ANS bound to liposomes of egg phosphatidylcholine or bovine serum albumin

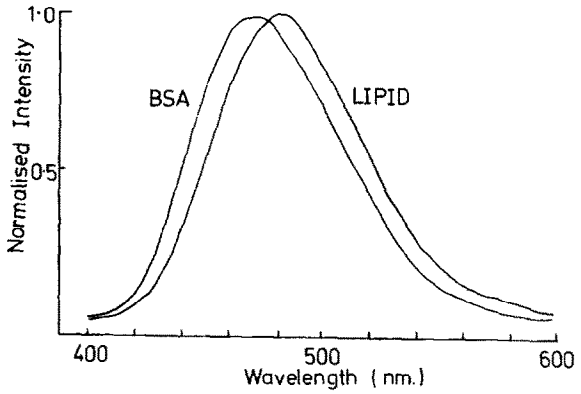

Fig.1. Total fluorescence emission spectra for ANS bound to liposomes of egg yolk phosphatidylcholine and bovine serum albumin, recorded on the laser-excited nanosecond spectrometer.

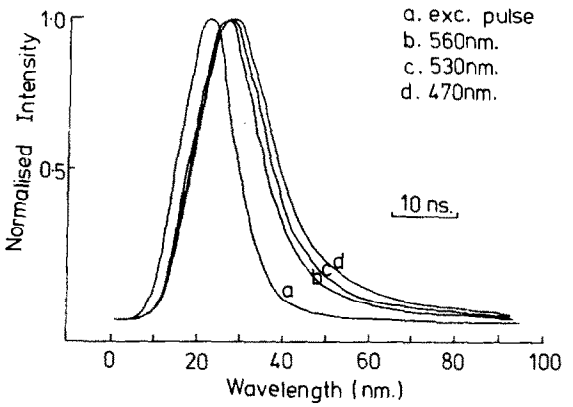

Fig.2. Fluorescence decay curves for ANS (1.7×10^{-5} M) bound to microsomal membranes (1 mg/ml), recorded at the wavelengths listed. Calibration 0.640 ns/channel.

can be well fitted to a single exponential, with fluorescent lifetimes listed in table 1. The fluorescent lifetimes measured using laser excitation are consistently shorter than those measured using a conventional N_2-flash lamp, probably because of effects associated with the polarization of the laser beam [11]. These reproducible differences do not affect the argument developed below.

Fluorescence decays for ANS bound to microsomal membranes can no longer be fitted to a single exponential and further, the fluorescence decays became longer with decreasing wavelength in the 470–750 nm region (fig.2). Measurements below 450 nm are complicated by significant blank fluorescence from the membrane with a maximum at about 340 nm and a broad tail extending to longer wavelengths (fig.3). The decays can be fitted with confidence to a double exponential (table 1) with components characteristics of lipid and protein bound ANS.

Fluorescence lifetimes decrease slightly with increasing ANS concentration, probably as a result of fluorescence quenching at high concentrations of bound ANS, as also observed in measurements of fluorescence intensity.

The significant differences in fluorescence lifetimes and emission maxima of the probe in the two environ-

Table 1
Fluorescence decay characteristics for ANS

Systems	Decay time (ns)	
	Laser excitation	N_2-flash lamp excitation
Egg-phosphatidylcholine	4.22 ± 0.1	7.14 ± 0.1
Bovine serum albumin	15.6 ± 0.1	16.7 ± 0.1
Microsomal membranes (1 mg protein/ml)		
ANS 6.3×10^{-6} M[a]	4.51 ± 0.4 (A^b = 0.056) 16.4 ± 0.8 (A = 0.017)	
ANS 5.2×10^{-5} M[a]	3.72 ± 0.4 (A^b = 0.065) 14.3 ± 0.8 (A = 0.019)	

[a] Data fitted to a double exponential, with a χ^2 statistic of 1.3
[b] Pre-exponential weighting factors

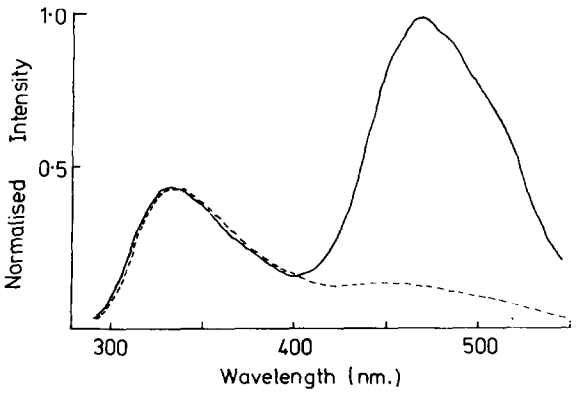

Fig.3. Total fluorescence emission spectra for ANS bound to microsomal membranes. (– – –) Fluorescence from blank microsome sample; (———) fluorescence from microsomes plus ANS (1.7×10^{-5} M).

ments allows one to isolate the emission from ANS bound to lipid and protein components by time-resolved emission spectroscopy. The spectral distribution of photons emitted coincident with the excitation pulse ($\Delta t = 0$, $\delta t = 2.5$ ns) will be weighted towards the component of fluorescence of shorter lifetime while with the time gate placed at a considerable delay after excitation ($\Delta t = 49$ ns, $\delta t = 3.2$ ns) the emission spectrum will arise solely from the long-lived component of fluorescence. The time-resolved spectra recorded are shown in fig.4.

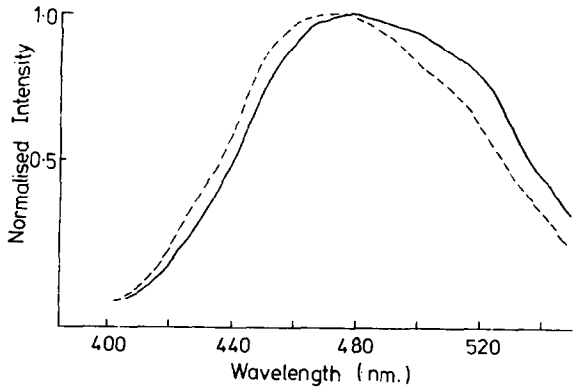

Fig.4. Time-resolved fluorescence emission spectra for ANS bound to microsomal membranes (conditions as fig.2). (——) Early gated spectrum; $\Delta t = 0$, $\delta t = 2.5$ ns; (– – –) late-gated spectrum; $\Delta t = 49$ ns, $\delta t = 3.2$ ns.

The early-gated spectrum has a fluorescence emission maximum the same as that for lipid-bound ANS whereas the component of long fluorescence lifetime has a spectrum comparable to that of ANS bound to BSA (compare fig.2,4). It has therefore been possible to resolve the spectra of ANS into two broad categories of binding site in the membrane, although the conventional steady-state fluorescence spectrum gives no indication of two components (fig.3). It is, of course, not possible to conclude from these results that only two distinct types of binding site exist, but the time-resolved spectra together with the fluorescence lifetime data do suggest that the majority of binding sites under these conditions do fall into the broad categories of lipid-like or protein (BSA)-like. From Scatchard plots of fluorescence intensity versus ANS concentration, the number of ANS binding sites in these membranes can be estimated to be 1.1×10^{-7}/mg membrane protein with an app. K_d 1.2×10^{-5} (J.R., A.G.L., D.C.W., unpublished observations). Varying the percentage occupancy from 30–95% produces no detectable changes in the relative occupances of the two types of site.

Having confirmed the assignment of the two components of the fluorescent decay curves for ANS binding to microsomes, it is now possible to use the fluorescent decay curves to study environmental effects of ANS binding. Figure 5 shows the effect of addition of increasing amounts of barbiturate. Clearly,

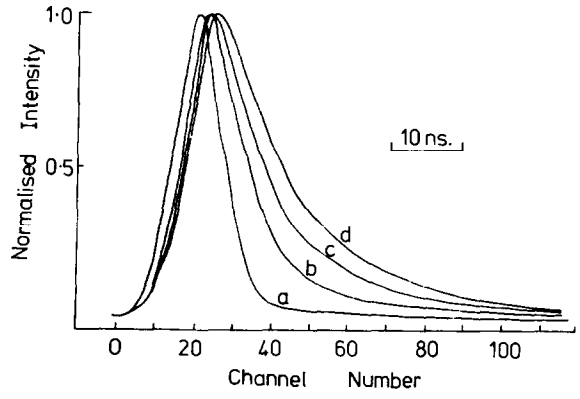

Fig.5. Fluorescence decay curves for ANS (1.7×10^{-5} M) bound to microsomal membranes (1 mg/ml) recorded at 480 nm, in the presence of pentobarbitone: (a) excitation pulse; (b) without pentobarbitone; (c) with 10^{-6} M pentobarbitone; (d) with 5×10^{-6} M pentobarbitone.

barbiturate causes an increase in fluorescence decay time for ANS bound to the membrane, attributable to an increased weighting for the longer-lived protein-bound component in the composite decay (table 1). Since steady state measurements show that addition of barbiturate leads to a decrease in fluorescence intensity, this means that barbiturate must preferentially displace ANS from its lipid binding site.

In conclusion, the technique of nanosecond time-resolved fluorescence spectroscopy is able to resolve spectra for fluorescent molecules bound in lipid and protein environments in a membrane, whenever the fluorescence emission maxima and fluorescence lifetimes differ significantly in the two environments. Because of the high pulse rate of the mode-locked cavity-dumped argon-ion laser system used, such spectra can be obtained typically in 30 min. Time-resolved fluorescence spectroscopy also, of course, has potentially important applications in other areas of biochemistry. Thus, for example, in proteins containing two Trp residues in different environments, fluorescence spectra for these two residues will be resolvable; such studies are underway with bovine serum albumin.

Acknowledgements

We thank the Science Research Council for a studentship to J. R. and for generous financial assistance.

References

[1] Ronai, A. and Wunderlich, F. J. (1975) J. Membr. Biol. 24, 381–399.
[2] Ghiggino, K. P., Phillips, D., Salisbury, K. and Swards, M. D. (1977) J. Photochem. 7, 141–146
[3] Daniel, E. and Weber, G. (1966) Biochemistry 5, 1893–1900.
[4] Vanderkooi, J. and Martonosi, A. (1971) Arch. Biochem. Biophys. 144, 87–98.
[5] Feinstein, M. B. and Felsenfeld, H. (1975) Biochemistry 14, 3041–3048.
[6] Freedman, R. B. and Radda, G. K. (1969) FEBS Lett. 3, 150–152.
[7] Yguerabide, J. (1973) in: Fluorescence techniques (Thaer, A. A. and Sernetz, M. eds) Springer-Verlag, Berlin, New York.
[8] Wang, H. H. (1978) personal communication.
[9] Easter, J. H., De Toma, R. P. and Brand, L. (1976) Biophys. J. 16, 571–583.
[10] Ghiggino, K. P., Phillips, D. and Lee, A. G. (1978) submitted.
[11] Spears, K. G., Cramer, L. E. and Hoffland, L. D. (1978) Rev. Sci. Instrum. 49, 255–262.