NANOSECOND TIME-RESOLVED EMISSION SPECTROSCOPY OF A FLUORESCENCE PROBE BOUND TO L-α-EGG LECITHIN VESICLES*

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SUMMARY

The monophoton counting technique was used to measure nanosecond time-resolved fluorescence emission spectra of 2-p-toluidino-naphthalene-6-sulfonate (2,6 p-TNS) adsorbed to lipid bilayer vesicles. A time-dependent red shift in the emission maximum was observed and the rate of this red shift was shown to be temperature dependent. Analysis of fluorescence decay curves obtained at different emission wavelengths indicates that the time-dependent spectral shifts are due to an excited-state reaction such as solvent relaxation or an excited-state interaction between the chromophore and a polar residue of the phospholipid.

INTRODUCTION

The advent of the monophoton counting technique for the measurement of fluorescence lifetimes has made it possible to obtain nanosecond time-resolved emission spectra with a timing resolution of better than 0.5 nanoseconds and a spectral resolution of better than 5 nm (1,2,3). Nanosecond time-dependent spectral shifts may be used to investigate a variety of excited-state reactions such as solvent relaxation, exciplex or excimer interactions, energy transfer or proton transfer provided that these processes occur during the time-span of the fluorescence emission.

N-arylaminonaphthalenesulfonates such as 2,6 p-TNS have been used as fluorescence probes to investigate a variety of biological systems including membranes (4). The fluorescence of these chromophores depends on the polarity of the solvent, being at lower energies in more polar solvents. The dependence of the emission maximum on polarity can be understood on the basis of excited-state solvent relaxation (4). Nanosecond time-dependent

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red shifts have been observed with 2,6 p-TNS dissolved in glycerol at 4°C where the relaxation time of the solvent is on the order of nanoseconds.

Huang (5) has described the preparation of homogeneous phosphatidylcholine bilayer vesicles and has shown (6) that they can adsorb 2,6 p-TNS. The aim of the present communication is to describe nanosecond time-resolved spectral studies with this model membrane system and to indicate that this new experimental approach may be of value in increasing our understanding of biological membranes.

MATERIALS AND METHODS

L-α-egg lecithin was obtained from Sigma, St. Louis, Mo., and purified by adsorption to activated silicic acid (UNISIL), by batch elution with chloroform-methanol mixtures and final elution with methanol as described by Hanahan (7). The purified lecithin was shown to run as a single spot on silica gel thin layer chromatography with two solvent systems (chloroform: methanol:water / 65:25:4 and chloroform:methanol:acetic acid / 7:3:1). Lecithin vesicles were prepared by sonication in 0.01M Tris-HCl, 0.1M NaCl buffer at pH 8.5 as described by Huang (5). The single bilayer vesicles were separated on Sepharose 4B (Pharmacia). Vesicle concentration was determined by a modified lipid phosphorus analysis from Bartlett (8). 2,6 p-TNS was prepared by Seliskar (9). All decay curves were obtained under single-photon counting conditions, and spectra were not corrected for non-linear response of the detection system.

RESULTS AND DISCUSSION

Fluorescence decay curves were collected at two to ten nanometer intervals between 390 nm and 530 nm. The decay curves at 390 nm and 530 nm are shown in Fig. 1 which also shows the profile of the lamp flash. The latter was obtained using a suspension of Ludox[1] as a scattering sample. Longer decay times were observed as the wavelength increases.

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[1] We thank H. Matthews of Dupont for kindly providing us with a sample of collidal silica, IBD-1019-69 (trade name Ludox).

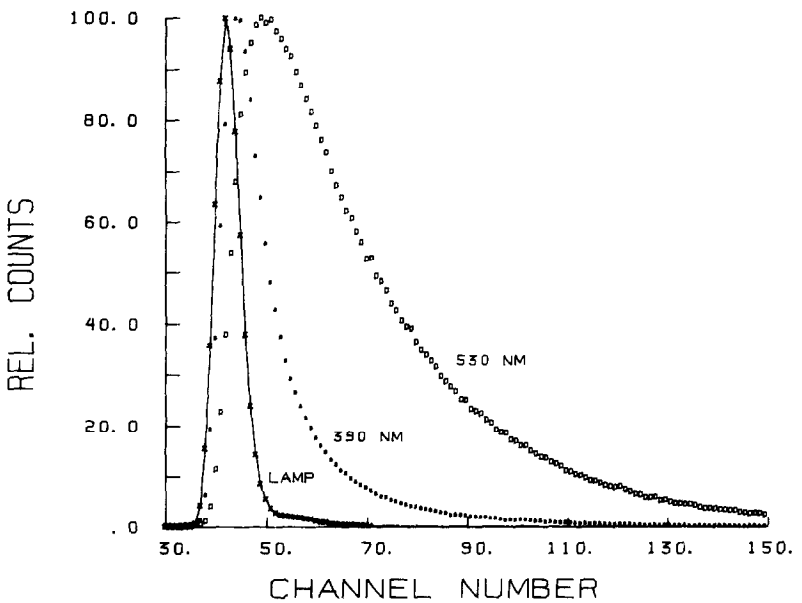

Fig. 1. Decay curves of 2,6 p-TNS adsorbed to L-α-egg lecithin bilayer vesicles at -2°C. 2,6 p-TNS (1 x 10^{-5} M); lipid concentration (1.5 moles lecithin/ml). Each decay curve was collected to 30,000 counts at the peak and there were 0.45 nsec/channel. The emission of the free dye is too low to be measured under these conditions.

Nanosecond time-resolved emission spectra generated from decay curves obtained at -2°C and 35°C are shown in Figs. 2A and B, respectively. Since only one channel was taken for each curve, the time-window is about half a nanosecond. For convenience, the peak of the lamp flash has been taken as zero time; the spectra at all times have been normalized to about the same intensity. A distinct red shift in the emission maximum with decay time is evident both at -2°C and 35°C. The rate of the red shift is greater at 35°C than at -2°C. It should be emphasized that in a non-viscous solvent, such as ethanol or water, the rate of any red shift is too rapid to be accurately measured by our instrument. The results shown in Fig. 2 are similar to the observations with 2,6 p-TNS dissolved in glycerol where a time-dependent red shift whose rate increases with temperature has been reported (2). The effect of temperature on the rate of the spectral shifts

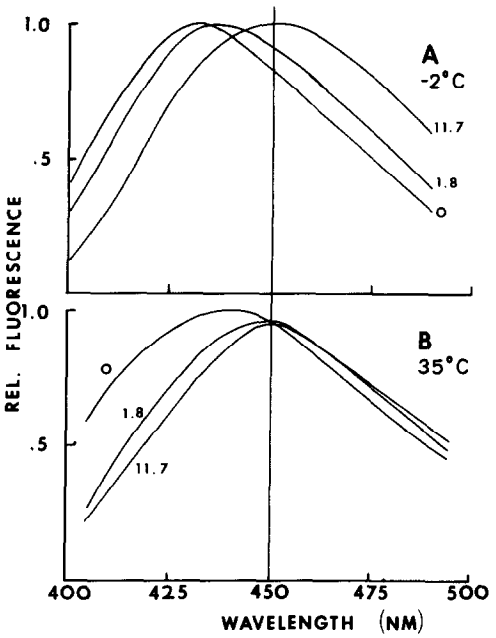

Fig. 2. Nanosecond time-resolved emission spectra of 2,6 p-TNS adsorbed to L-α-egg lecithin vesicles. Concentrations as in legend to Fig. 1.

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can be seen more clearly in Fig. 3 which indicates the emission maxima of the time-resolved spectra as a function of decay time at different temperatures. It appears that the spectra converge to the same emission maximum at long times at all temperatures. At the present time no statement can be made regarding the emission maximum at the real time-zero since this is ill defined due to the experimental convolution of the decay curve with the lamp flash.

Although time-resolved spectral shifts of the type described here can usually be attributed to an excited-state reaction, we have shown that a mixture of emitting species with appropriate spectral and decay properties can give rise to "trivial" spectral shifts. Loken (10) has shown that analysis of the exponential character of the decay curves obtained at different emission wavelengths can be used to distinguish these two cases. A mixture of two species (which would arise if the vesicles have two types of

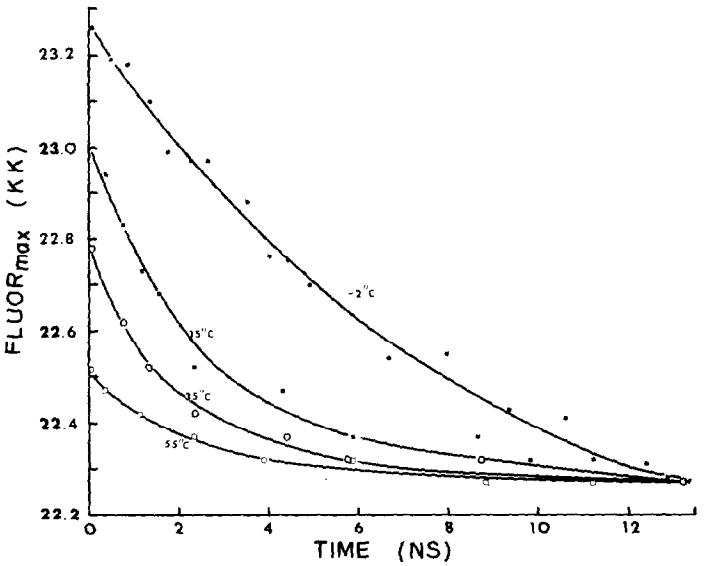

Fig. 3. Time-resolved emission maxima of 2,6 p-TNS adsorbed to L-α-egg lecithin vesicles as a function of time and temperature. Emission maxima are plotted as energy in kilokaysers (kK = 10^3 cm^{-1}). Conditions as in legend to Fig. 1.

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binding sites for 2,6 p-TNS) would give rise to double exponential decay with one decay predominating at the blue edge of the emission and the other at the red edge. In contrast, a simple two-state mechanism predicts the following pattern in going from the blue edge of the emission towards the red: single exponential decay → double exponential decay → single exponential decay → and finally at the far red edge of the emission, double exponential decay with equal and opposite amplitude factors. The two decay times should remain the same. The decay curves obtained at each wavelength for the TNS-vesicle system were analyzed for two exponentials by the method of moments (11). The results of this analysis are presented in Table I. At 390 nm the decay approaches a single exponential with a decay-time of 1.5 nsec. From 410 nm to 451 nm two exponential terms are required to fit the data. At 470 nm single exponential decay is observed and at 530 nm the decay approaches a double exponential with equal and opposite amplitude terms.

Table I. Method of moments analysis of fluorescence decay curves

Wavelength	α_1	τ_1	α_2	τ_2	α_1/α_2
390 nm	.246	1.5 nsec	.028	8.2 nsec	8.88
410 nm	.805	2.3 nsec	.275	9.6 nsec	2.93
430 nm	.830	3.8 nsec	.630	10.8 nsec	1.32
451 nm	.639	7.2 nsec	.456	12.3 nsec	1.40
470 nm	.001	--------	.706	10.5 nsec	0.001
490 nm	-.116	8.0 nsec	.623	10.5 nsec	-0.186
515 nm	-.103	0.97 nsec	.166	10.8 nsec	-0.620
530 nm	-.091	0.66 nsec	.10	11.3 nsec	-0.914

CONCLUSIONS

Nanosecond time-resolved spectral shifts are observed in the fluorescence emission of 2,6 p-TNS adsorbed to L-α-egg lecithin vesicles. Analysis of the decay curves at different emission wavelengths is not consistent with the "trivial" interpretation involving different types of binding sites, but indicates that an excited-state reaction is taking place. Although the two-state mechanism discussed above is probably an oversimplification, it is evident that the analysis of the data is consistent with the concept of an excited-state reaction. Since similar spectral shifts are observed with 2,6 p-TNS in a viscous solvent such as glycerol but not in a non-viscous solution such as ethanol or water, the results are interpreted in terms of an excited-state reaction between the probe and a polar moiety with restricted mobility. Additional expeiments will be required to determine whether the polar group is a solvent molecule or a charged portion of the lecithin. Nanosecond time-resolved emission spectroscopy is a new technique capable of providing information about the dynamic structure of biological membranes.

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