protein. If the transducer protein transports an ion down the field gradient, the system is electro-mechanically coupled.

The theory predicts that the action potential is a thinning (Na transport) followed by a thickening (K transport) of the bilayer; that the proton pumping of bacteriorhodopsin and other transport phenomena occur as standing compaction waves; and that rhodopsin, when it absorps a photon, generates a wave (perpendicular to the plane of the bilayer) spreading to the edge of the disc that contains it.

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RESOLUTION OF HETEROGENEOUS FLUORESCENCE BY PHASE-SENSITIVE FLUORESCENCE SPECTROSCOPY

JOSEPH R. LAKOWICZ AND HENRYK CHEREK
Department of Biological Chemistry, University of Maryland, School of Medicine, Baltimore, Maryland 21201 U.S.A.

Fluorescence spectroscopic methods are widely utilized in studies of proteins, membranes and more complex biological samples. One hindrance to the interpretation of these data is the heterogeneous nature of the fluorescence emission. Frequently, emission occurs from more than a single fluorophore, e.g., the emission from tyrosine and tryptophan residues in proteins (1, 2). We report here the development of a new method, phase-sensitive fluorescence spectroscopy (PSFS), which allows the emission spectra of each fluorophore in a two-component mixture to be recorded directly. This method uses a phase fluorometer, modified by the addition of a lock-in amplifier. Emission spectra are recorded at various detector phase angles. The chosen angles can be out of phase with any given component, thereby suppressing the emission from this component. The lifetime of the suppressed component may be calculated from the detector phase angle. Phase-sensitive fluorescence spectroscopy can thus resolve the spectra or lifetimes of a heterogeneous sample. In this report we describe our studies with mixtures of fluorophores that simulate the heterogeneous emission from proteins.

THEORY

The sample is excited with light whose intensity is modulated sinusoidally

$$I(t) = 1 + m_I \sin \omega t \qquad (1)$$

where ω is the circular modulation frequency and m_I is the degree of modulation. The emission is then

$$F(t) = 1 + m_F \sin (\omega t - \phi) \qquad (2)$$

where m_F is the modulation of the emission and ϕ is the phase shift (3). This shift is related to the fluorescence lifetime (τ) by

$$\tan \phi = \omega\tau. \qquad (3)$$

When quantified using a lock-in amplifier, the modulated emission yields a DC signal which depends upon the phase angle of the detector (ϕ_D)

$$F(\phi_D) = k \cos (\phi_D - \phi). \qquad (4)$$

The constant k contains factors due to concentration, lifetime and other molecular and instrumental parameters.

Suppose the sample contains two fluorophores, A and B, with different lifetimes, τ_A and τ_B, and spectral distributions, $I_A(\lambda)$ and $I_B(\lambda)$. The modulated emission then consists of two sine waves of the same frequency but different phase angles (Eq. 3). Phase-sensitive detection yields

$$F(\phi_D) = k_A I_A(\lambda) \cos (\phi_D - \phi_A) + k_B I_B(\lambda) \cos (\phi_D - \phi_B) \qquad (5)$$

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where the constants k_i depend on relative concentrations and the factors described above. The detector phase can be chosen to be 90° out of phase with either ϕ_A or ϕ_B. Then the emission spectrum results solely from the unsuppressed component. Thus, if the lifetime of either component is known, its emission can be suppressed. Then the emission spectrum of the other component can be recorded from the mixture. Alternatively, if the steady-state emission spectrum of one component is known, the detector phase ϕ_D can be varied until the phase-sensitive emission spectrum superimposes on the steady-state spectrum. Then the detector phase angle may be used to calculate the lifetime of the suppressed component.

MATERIALS AND METHODS

Phase-sensitive detection of fluorescence was performed on a phase fluorometer similar to that described previously (3), modified by addition of a lock-in amplifier (Model 5204, Princeton Applied Research Corp., Princeton, NJ)(4). The lower frequency cross-correlated signals (3) were used for phase sensitive detection. Steady-state emission spectra were obtained using this same instrument with the light modulator turned off to provide constant illumination. Fluorescence lifetimes were measured as described previously using *p*-terphenyl as a reference (5). This reference fluorophore was also used to determine the phase of the excitation beam as measured with the lock-in amplifier (4).

Indole and 2,3-dimethylindole (DMI) were dissolved in dodecane and purged with argon to remove dissolved oxygen. *N*-acetyl-L-tyrosinamide (NATrA) and *N*-acetyl-L-tryptophanamide (NATpA) were dissolved in 0.025 M Tris chloride buffer, pH 7.5. All experiments were performed at 20°C. Indole and DMI were excited at 288 nm and NATrA and NATpA were excited at 280 nm. The emission bandpass was 8 nm.

RESULTS

To model the emission from proteins containing more than a single tryptophan residue we chose a mixture of indole and 2,3-dimethylindole (DMI). Their steady-state emission spectra are different (Fig. 1) and their lifetimes were determined to be 10.6 and 3.0 ns, respectively. We recorded phase-sensitive fluorescence spectra from the mixture. The phase-sensitive spectral distribution of the mixture depends upon the detector phase angles, whereas these spectra are independent of phase angle for the pure solutions[1]. Importantly, the detector phase can be chosen so that the spectrum of the mixture superimposes on the steady-state spectra of each pure species (Fig. 1). When $\phi_D = 24° + 90°$, the spectrum matches that of indole. A phase angle of 24° (at 30 MHz) corresponds to a lifetime of 2.4 ns, which is comparable to that measured for DMI. When $\phi_D = 63° - 90°$ the spectrum matches that of DMI. A phase angle of 63° corresponds to a lifetime of 10.4 ns, which is comparable to the measured lifetimes of indole. The sign of the 90° phase shift determines only whether the phase sensitive signals are positive or negative.

We also examined a mixture of NATrA and NATpA, in this case to model the emission from proteins which

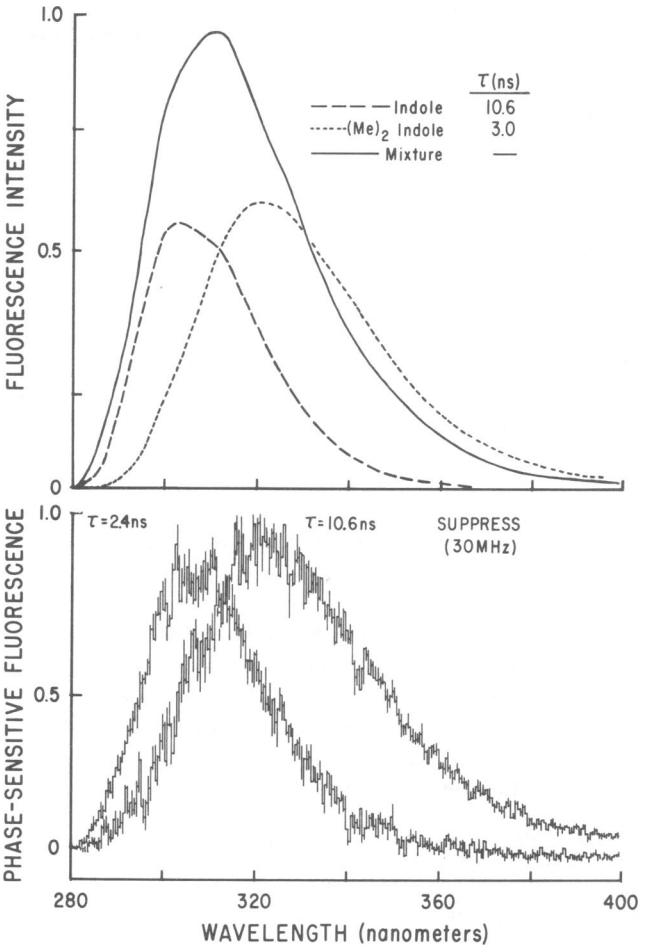

FIGURE 1 Fluorescence emission spectra of indole, dimethylindole and the mixture. Steady-state emission spectra are shown for indole (---), dimethylindole (·····) and the mixture (—). Also shown are phase sensitive emission spectra of the mixture. Detector phase angles of 24° + 90° and 63° − 90° correspond to suppression of DMI (τ = 2.4 ns) and indole (10.4 ns), respectively. The modulation frequency was 30 MHz.

contain both tyrosine and tryptophan residues. Steady-state emission spectra of each fluorophore and the mixture are shown in Fig. 2; phase sensitive fluorescence spectra of the mixture are shown in Fig. 3. Clearly, when the detector phase is chosen to suppress a component with τ = 2.8 ns (NATpA) the spectrum matches that of NATrA. When ϕ_D is chosen to suppress τ = 1.8 ns (NATrA) the spectrum matches that of NATpA. Thus, if the steady-state emission spectra are known, or can be estimated, the lifetimes of each component in a mixture may be determined.

The signal-to-noise ratio of the phase-sensitive spectra of the NATrA/NATpA mixture is rather poor, for two reasons. First, excitation was at 280 nm and the output of our light source is weak at this wavelength. Second, the lifetimes of NATrA and NATpA differ by only 1 ns. Suppression of each component results in attenuation of the amplitude of the remaining component to $|\sin(\phi_A - \phi_B)|$ of the original intensity. In spite of these difficulties we were able to resolve the emission of NATrA and

[1]Lakowicz, J. R., and H. Cherek. 1981. Resolution of heterogeneous fluorescence from proteins and aromatic amino acids by phase sensitive detection of fluorescence. *J. Biol. Chem.* 256:6348–6353.

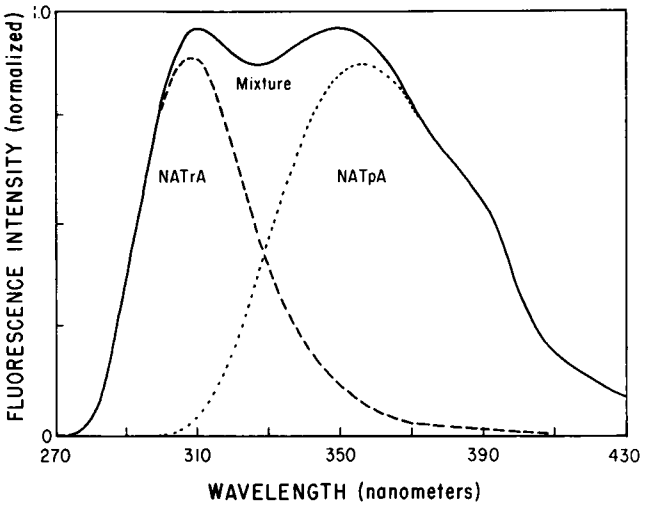

FIGURE 2 Fluorescence emission spectra of NATrA, NATpA and the mixture.

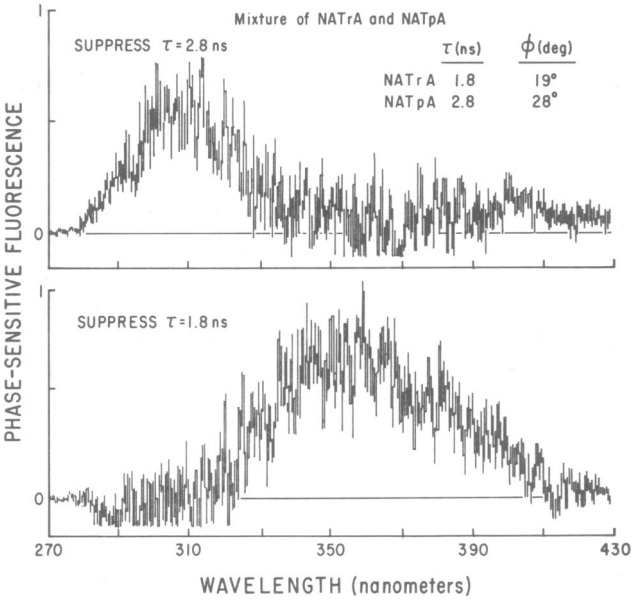

FIGURE 3 Phase-sensitive emission spectra of the NATrA-NATpA mixture. The detector phase was adjusted to suppress $\tau = 2.8$ ns for the upper panel and $\tau = 1.8$ ns for the lower panel. The modulation frequency was 30 MHz.

NATpA, which indicates the high potential resolving power of phase sensitive fluorescence spectroscopy. More recent studies using improved optics indicate that still higher resolution is obtainable. It appears that the limiting factor in resolution will be the intrinsic heterogeneity of individual fluorophores rather than instrumental factors.

DISCUSSION

Heterogeneous fluorescence from proteins has been resolved using other methods such as selective excitation of tryptophan residues (6) by quenching (7) and by chemical (8) and biological (9) modification. Selective excitation usually cannot be used to resolve tryptophan residues, and none of these methods reveals directly the individual lifetimes. PSFS has the potential to reveal both spectra and lifetimes, and thus provides a useful additional method for analysis of the fluorescence from complex samples. Moreover, excited-state processes can reveal the dynamic properties of proteins and membranes (10) and phase-sensitive methods will be useful in quantifying these reactions.

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