PICOSECOND RELAXATION OF THE FLUORESCENCE PROBES ANS AND TNS IN AQUEOUS SOLUTIONS

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1. Introduction

Fluorescent dyes whose quantum yields are particularly sensitive to their environment have been extensively used as probes in structural studies of biological species [1]. Interpretation of the emission properties is used to give information on structure and relaxation dynamics. Dyes of the *N*-arylaminonaphthalene sulfonate family have been widely applied as probes of the polarity of sites in proteins or as hydrophobic probes since the fluorescence of these molecules in non-polar solvents varies dramatically from that in polar solvents [2,3]. The fluorescence properties of these organic dyes covalently linked to proteins has been shown to depend upon the conformational state of the protein [4]. Generally, the most common feature of the fluorescence examined is the quantum yield. However, interest now is being directed towards understanding the kinetics of many of the processes in biological specimens following excitation with a short burst of energy. Therefore, time-resolved emission of the fluorescence is essential for an indication of the conformational changes in the specimen following excitation, solvent reorientation, complex formation or energy transfer processes, which straightforward measurement of quantum yield is incapable of providing. To understand these processes, it is first needed to examine the fluorescence decay of the fluorescence probes themselves following excitation. In the past, information of this type has been rather limited. This is due to the fact that many of the probes in aqueous solutions for example have extremely low quantum yields [5] and ultrafast (~ps) decays, making accurate measurement difficult. However, our recently developed synchroscan streak camera used in conjunction with a low average power CW mode-locked dye laser, enables picosecond lifetimes to be examined routinely with a resolution of ~3 ps.

Here, we report on the measurement of the decay kinetics of the commonly used fluorescence probes, determining their radiative and non-radiative decay rates and examining these decays in various mixed solvent solutions of variable viscosity and polarity.

2. Materials and methods

The fluorescent probe dyes 8-anilino-1-naphthalene-sulfonic acid (ANS), as the magnesium salt, and 2-*p*-toluidinylnaphthylene-6-sulfonate (TNS) as the potassium salt, were obtained from Sigma and were used without any further purification. All the solvents were spectroscopic grade and were used as obtained from BDH Ltd. Where mixed solvents were used, the mixture was placed in a vibration bath for some time prior to use to ensure proper homogeneity of the solution.

The experimental arrangement is an improvement but similar to that in [6]. The source of picosecond pulses used to excite the fluorescence was derived from the intracavity second harmonic frequency generated from a CW mode-locked Rhodamine 6G dye laser [7]. Time-resolved detection of the generated fluorescence was recorded on the synchroscan streak camera [8]. The experimental arrangement is shown schematically in fig.1. As the individual components have been detailed in [6–8], only a brief description of the technique will be given here.

The second harmonic was generated by placing a 1.5 mm thick crystal of ADP at the common focal point and centre of curvature, respectively, of 2 mirrors placed in the cavity of a synchronously pumped

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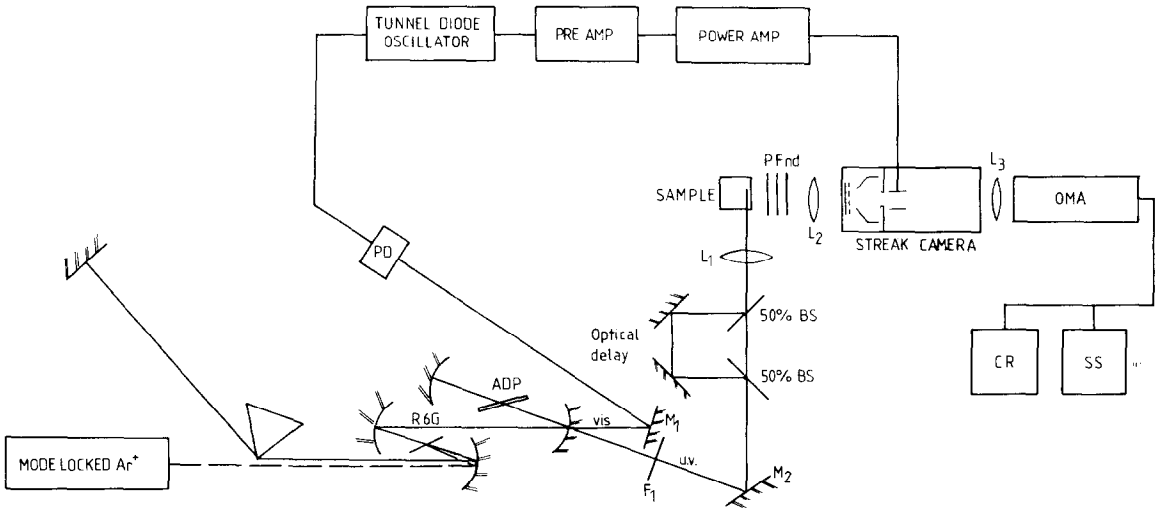

Fig.1. Experimental arrangement.

jet stream CW Rhodamine 6G dye laser. Typically, the output in the UV was tunable from 290 nm–308 nm and consisted of a train of pulses of ~2 ps in duration occurring at a 70 MHz repetition rate. The average power in the UV beam after passing through the filter F_1 to remove any visible radiation was 1.5 mW (peak power/pulse ~10 W). This was directed via mirror M_2 through an optical delay line which provided a time calibration mechanism and a linearity check for the streak camera. For the fluorescence decay measurements, only one beam was used. The UV was focussed using lens L_1 into the 10 mm square cross section cell containing the sample under investigation. A maximum excitation power density of 30 kW/cm^2 could be achieved at the sample. The fluorescence was detected at right angles with respect to the incident beam and was passed through a polarizer P set at 54.7° to the incident polarization to remove any distortion due to fluorescence depolarization [9] and a filter F to transmit only the fluorescence waveband. In all cases, the level of the fluorescence signal was so high as to saturate the detection equipment and ND filters had to be inserted to lower the signal to a recordable level. A lens L_2 focussed the signal on to the photocathode of the streak camera. Derivation of the streak mechanism was achieved by directing the visible light which leaked through the nominally 100% reflecting laser cavity mirror, on to a photodiode (PD). The generated electrical signal triggered a tunnel diode oscillator in synchronism with the driving pulses. Following stages of amplification, this sinusoidal voltage ramp was applied to the streak plates. Synchronization of the fluorescent event and deflection voltage are easily achieved since both are derived from the same laser pulses. The synchroscan streak camera operates such that the streaked images are accumulated at the repetition rate of the excitation pulses deriving the fluorescence signal (in this case, 7×10^7/s) and are precisely superimposed with a jitter of <1 ps. In such a manner, extremely weak fluorescence signals can be detected for low levels of excitation power, so eliminating non-linear effects in the samples. An optical multichannel analyser (OMA) detected the streaked image on the phosphor of the streak camera and these were recorded and stored for subsequent display on a chart recorder CR, or storage scope SS.

3. Results and discussion

All fluorescence decay measurements were taken for 10^{-4} M solutions of ANS and TNS, although variations over 10^{-3}–10^{-6} M gave rise to no apparent change in the measured fluorescence lifetime. Some typical decay curves for TNS in various water/ethanol mixtures are shown in fig.2. A much more rapid lifetime variation to ethanol content was shown in TNS in comparison to ANS and consequently is the more sensitive probe for solvent or environment studies.

The fluorescence lifetimes assuming a single exponential decay are indicated on fig.2. The vertical line to the left of fig.2c is caused by the limited spatial extent of the detection head of the OMA. In most cases, the fluorescence lifetimes were not measured beyond a recorded width of 1.5 ns. This is due to the fact that the synchroscan measurements in the picosecond regime are more accurate than those in the nanosecond region [10] due to deviations from linearity of the sweep, giving rise to errors greater than an acceptable 5%. Over the range from 100% water to a 60% water content in a water/ethanol mixture, the lifetime of TNS increased from 78 ps to 1171 ps.

The similar trend for ANS is shown in fig.3 where the fluorescence lifetime increased from 262 ps to 1550 ps for an identical range of solvent mixture. Also shown in fig.3 are the derived radiative decay rate k_R and non-radiative decay rate k_{NR}. These were calculated from the measured fluorescence decay times and the fluorescence quantum yields in [3,11]. In the derivation of these rates, it was assumed that the fluorescence decays behaved as simple single exponentials. It can be seen that over the range of solvent mixture, the radiative decay rate decreased slowly

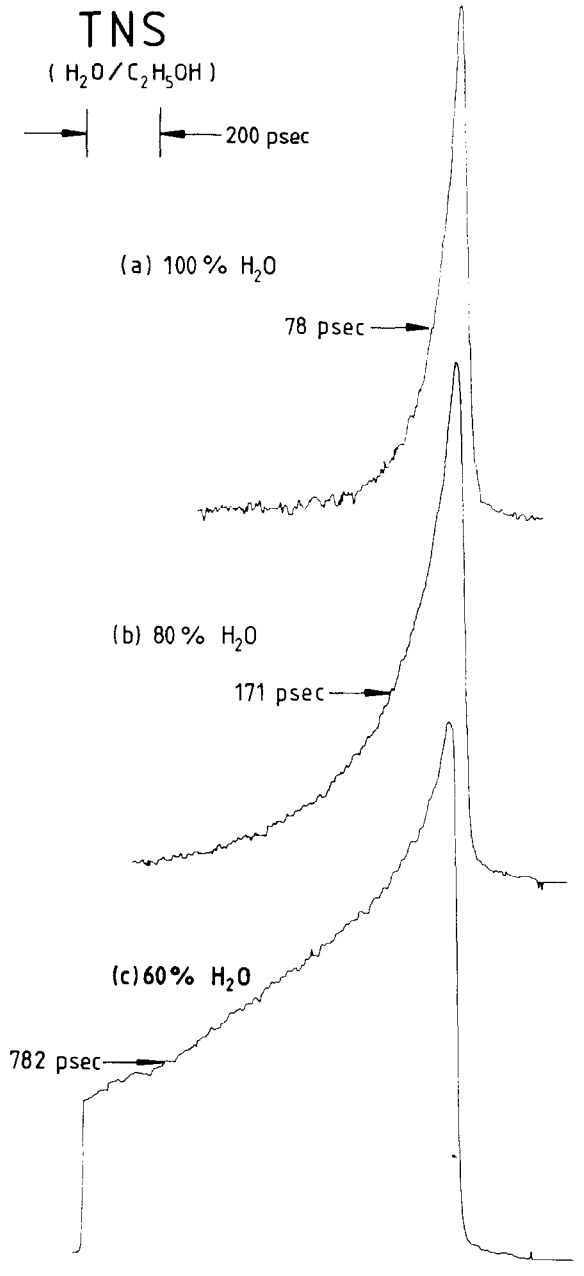

Fig.2. Typical fluorescence decay curves of TNS in: (a) 100% water; (b) 80% water; (c) 60% water–ethanol solvent mixtures. The 1/*e* lifetimes are indicated.

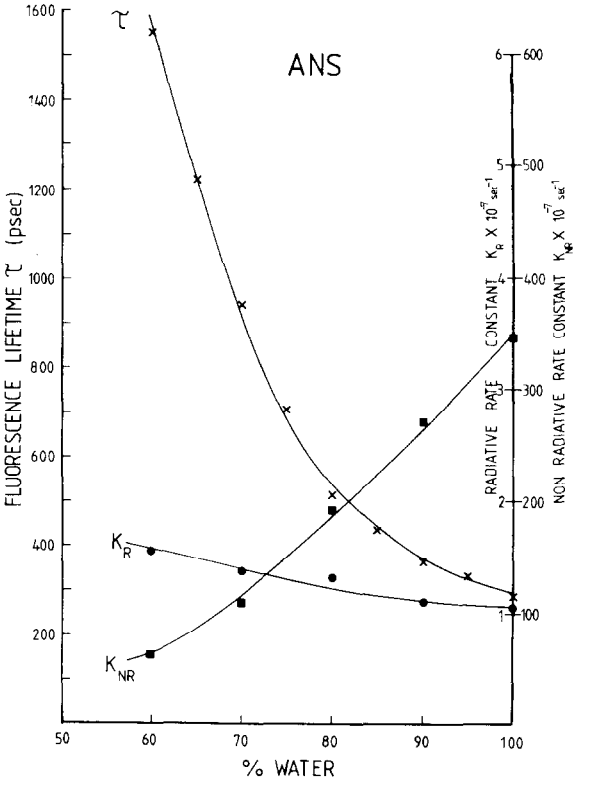

Fig.3. Variation of the fluorescence lifetime τ, the radiative rate constant k_R and the non-radiative rate constant k_{NR} with percentage water content (v/v) of ANS in water/ethanol mixed solvents.

from 1.55×10^7 s^{-1} to 1.04×10^7 s^{-1} while the non-radiative rate showed practically a 6-times increase in its value from 62.9×10^7 s^{-1} to 346.2×10^7 s^{-1} with increasing % water content up to 100%. Nanosecond absorption measurements [12] have shown that the solvated electron is a product of photoexcited ANS in solution, and in polar solvents it has been proposed that photoionization would be the major deactivation process of the excited singlet. On the assumption that this ionization proceeds via a charge-transfer-to-solvent state, and that k_R is independent of temperature, then:

$$1/\tau = k_R + A, \exp(-E/RT)$$

where τ is the fluorescence lifetime, T the solution temperature, E the activation energy for the electron transfer and A, a frequency factor. The latter two constants can be determined by measuring the variation of fluorescence lifetime with temperature. By contacting the sample cell to a Peltier junction, this was examined. However, over a range of 30°C, no detectable difference in the fluorescence lifetime was observed. This indicates that the transfer to the charge-transfer-to-solvent state is extremely rapid and requires no activation energy.

A distinct solvent deuterium effect was recorded for the fluorescence lifetimes, as has been reported for quantum efficiency [13]. In D_2O, the fluorescence lifetimes increased to 127 ps and 436 ps for TNS and ANS, respectively. It had been accepted that these increases on deuteration have been due to the O–D stretching vibatration being less efficient in dissipating any excess energy. However, other factors such as charge-transfer-to-solvent or localized solvent cage reorientation may also contribute considerably.

Where mixed solvents are involved, it may be possible at any one instant for several different solute–solvent environments to exist. Since the probe molecules ANS and TNS are so sensitive to the local environment as above, each different site should give rise to a differing emission. Where time-integrated results are observed, e.g., quantum efficiency such differences will not be resolvable, the result representing an average for the total or average environment. However, with time resolved measurements, such variations in the emission should be detectable. In water, semilogarithmic traces of the fluorescence decay of ANS showed within experimental error a single exponential decay, while in mixed solvents (water/ethanol) deviations from single linearity were observed. These deviations reinforced the idea of differing solvent–solute sites interacting and giving rise to differing rate kinetics of deexcitation which consequently manifest themselves in an overall decay rate which cannot be represented by a single exponent. Naturally, this introduces errors into the derivations of the radiative and non-radiative decay rates determined in fig.3. However, division into several components would be difficult and the results are more representative of an average for the solvent interaction. It would be necessary to spectrally resolve separate wavelength regions corresponding to the various shifts in the fluorescence due to different solvents to further clarify this problem.

Other factors apart from polarity are important in the relaxation processes of many of these fluorescence probes in solution, for example, deuteration, hydrogen bonding or viscosity [5]. The decay of TNS (10^{-4} M) in various solvent mixtures was examined (fig.4). In the more viscous mixtures, i.e., ethanediol or glycerol, the fluorescence lifetime is considerably reduced. The increased viscosity seems to play an important role since at room temperature 60% by volume mixed solutions with water of glycol and acetonitrile have dielectric constants of 67.5 and 67.4,

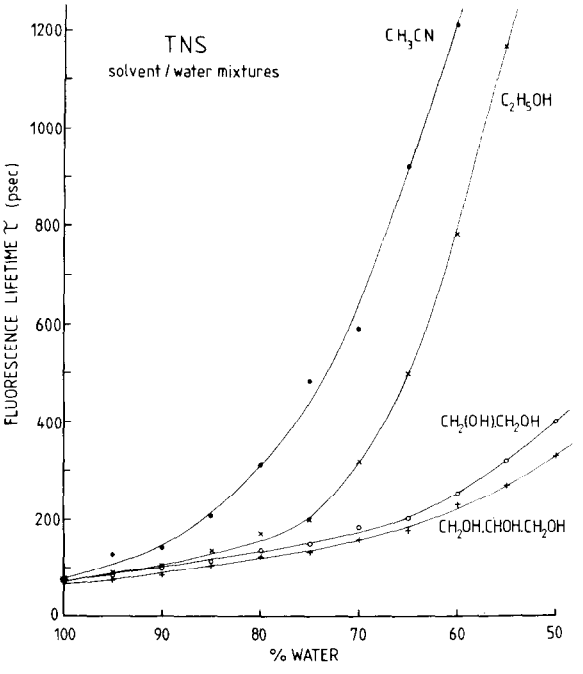

Fig.4. Variation of fluorescence lifetimes of TNS with percentage water content by volume for solvent mixtures of water with acetonitrile, ethanol, ethanediol and glycerol.

respectively, while the corresponding fluorescence lifetimes are 242 ps and 1213 ps. For a given percentage water content in fig.4 the trend is for an increase in the measured fluorescence lifetime for decreased viscosity, in going from glycerol, to ethanediol to ethanol to acetonitrile. Some other competing mechanism in the excited state is possible therefore in more viscous solutions. This is most likely occurring at the solvent shell relaxation mechanism, which increased viscosity giving rigidity enabling other processes, e.g., inter-system crossing to triplet to occur, and these competing channels of relaxation can reduce the fluorescence lifetime. In the more viscous solvent mixtures, semi-logarithmic traces of the decay showed a good linear decay following a short initial non-linear region.

Thus, in a mixed solvent or mixed polarity environment, the time-resolved emission of fluorescent probes is difficult to fully interpret, inhomogeneties in the solutions being resolved. In addition to polarity, other environmental-sensitive, non-radiative processes do occur and are reflected in the modifications to the fluorescence decay profiles. In time-resolved studies of conformational changes in proteins or other biological species under the influence of ultrashort radiation burst, all of these processes must be considered in explanation of the relaxation processes and micro- and macro-environmental effects considered. However, the synchroscan streak camera and the frequency doubled CW mode-locked dye laser should provide a useful tool in studies of this type.

Acknowledgement

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