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TORSIONAL REARRANGEMENTS OF ARYL-SUBSTITUTED ANTHRACENES MEASURED BY PHASE FLUOROMETRY

Gabor LACZKO * and Joseph R. LAKOWICZ **

University of Maryland, School of Medicine, Department of Biological Chemistry, Baltimore, MD 21201, U.S.A.

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We examined the fluorescence phase angle spectra of aryl-substituted anthracenes in viscous solvents. These phase angles reflect the wavelength-dependent lifetimes displayed by the fluorophores. At intermediate viscosities the phase angles increased and decreased in accordance with the valleys and peaks of the structured emission spectra. At lower and higher temperatures the phase angles were independent of emission wavelength, which is equivalent to decay times which are independent of emission wavelength. We attribute the wavelength-dependent phase angles to rotation of the unsaturated side chains towards a conformation more coplanar with the anthracene ring. By comparative studies with 9,10-di(α-naphthylanthracene), 9,10-diphenylanthracene, and 9-vinylanthracene we showed that lower temperatures are required to inhibit rotation of the smaller phenyl and vinyl substitutents. Since these excited-state rearrangements are sensitive to the motional freedom allowed by the surrounding solvent, we suggest that this phenomenon may serve as a probe of volume fluctuations in macromolecules.

1. Introduction

Fluorescence methods are widely utilized to investigate the dynamic properties of biological macromolecules [1]. Its usefulness originates with the lifetimes of the excited states, which are typically near 10 ns. During this time a number of molecular processes may occur which alter the fluorescence spectral properties that are observed. For example, the fluorophore may undergo rotational diffusion, and the angular displacement is revealed by the decrease in fluorescence polarization. The magnitude of the depolarization is often used to estimate the apparent viscosity encountered by the fluorophore [2,3]. Alternatively, the fluorophore may be sensitive to the polarity of its surroundings and to the rate at which the polar groups in this environment reorient around the excited state. The rate of reorientation is revealed by the rate at which the emission spectra shift to longer wavelengths [4]. In this report we describe a different excited-state process which can potentially reveal the extent of volume fluctuations in macromolecules. This process is torsional rotation of a vinyl or aromatic substituents which are attached to an anthracene ring system. In the ground state these side groups do not appear to be in electronic conjugation with the anthracene, and are thus noncoplanar. However, in the excited state, these rings appear to rotate towards the coplanar orientation. We can detect this excited-state process from the wavelength-dependent phase angles of the fluorescence emission, i.e., the phase-angle spectra. The phase-angle spectra reflect average durations of the excited states which emit at each

Abbreviations: 9-VA, 9-vinylanthracene; DPA, 9,10-diphenylanthracene; DANA, 9,10-di(α-naphthyl)anthracene; DMP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl]benzene; 9-MA, 9-methylanthracene.

* On leave from Jozsef Attilla University, Szeged, Hungary.

** To whom reprint requests and correspondence should be sent.

wavelength. In the presence of overlapping emissions and/or excited-state processes it is difficult to interpret the apparent lifetimes measured using either the pulse or the phase method. Nonetheless, the phase-angle spectra indicate that the extent of the torsional rearrangements appear to be sensitive to both solvent viscosity and the size of the rotating unit.

2. Materials and methods

All fluorescence phase measurements were performed using a commercial phase fluorometer, similar to that described previously [5]. The modulation frequency was 30 MHz. Because the measured phase-angle differences across the emission spectra were small it was necessary to correct carefully for the wavelength- and/or geometry-dependent time response of the photomultiplier tubes. This was accomplished as described previously [6], except for the following modifications. The lifetime of DMP in ethanol was assumed to be 1.45 ns, irrespective of emission wavelength. The measured phase angles across the emission spectrum of DMP are shown in fig. 1. One notices that these angles not quite constant with emission wavelength. Instead, the phase angles decrease gradually with increasing wavelength. This decrease is approximately linear with wavelength. A similar wavelength-dependent decrease in the phase angle was found for 9-MA (fig. 1) and was found previously for a number of fluorophores whose decay is thought to be a single exponential and to be independent of emission wavelength (ref. 6, and references therein). A 'color effect' is expected to yield phase angles which increase with emission wavelength. Hence, it seems likely that the wavelength-dependent decrease is due to some other instrumental effect, the nature of which is unknown at present.

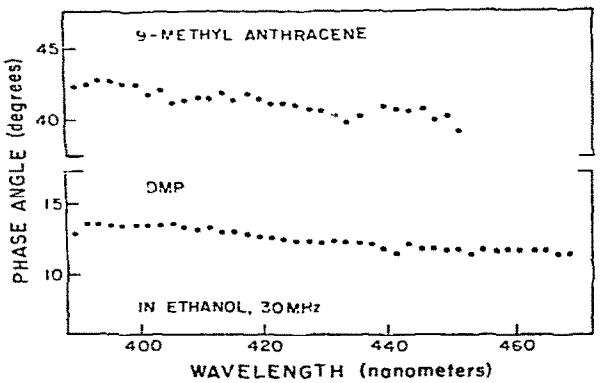

Fig. 1. Dependence of the measured phase angles on emission wavelength for 9-MA and DMP.

The wavelength-dependent response of our instrument is stable with time and small. Nonetheless, a correction procedure was developed which corrected for this minor effect. We needed to scan the phase of the emission in a continuous manner, without measuring the phase of the reference at each wavelength. The emission of the sample was scanned repeatedly, and the measured phase angles at each wavelength were averaged by a computer. Typically, 10–40 scans were averaged for each sample. After each five scans we returned to the reference sample (DMP) and measured its phase angle at 420 nm. The reference phase angle at this wavelength was assumed to be 15.29° (1.45 ns at 30 MHz). This value was added to all the wavelength-dependent phase angles of the sample, which were measured relative to DMP at 420 nm. The measured phase angles were additionally corrected for the wavelength-dependent response of the instrument by subtracting a wavelength-dependent correction factor $\phi_c(\lambda)$, given by $\phi_c(\lambda) = (420\ \text{nm} - \lambda) \times 0.0395$ deg./nm. This factor is derived by drawing a straight line through the wavelength-dependent phase angles shown in fig. 1, and by assuming the measured phase angle of the reference to be correct at 420 nm.

All sample solutions were in propylene glycol. Polarizers were not used in either the excitation or the emission light paths. The excitation and emission band-passes were each near 4 nm, the excitation wavelengths were 370 nm for 9-VA, and 375 nm for DPA and DANA. The absorbances at the excitation wavelength were near 0.4.

3. Results

Steady-state emission spectra of DANA in propylene glycol are shown in fig. 2. Propylene glycol

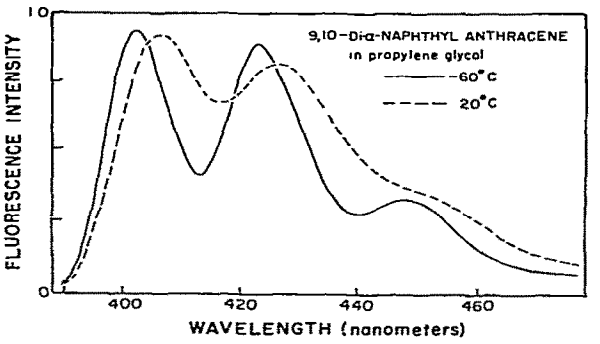

Fig. 2. Emission spectra of DANA in propylene glycol.

was the chosen solvent because its viscosity increases dramatically at low temperatures, and it forms an optically clear glass. At low temperature (−60°C) the emission spectrum displays vibrational structure comparable to that found for anthracene. Some vibrational structure is also seen at high temperature (20°C), but the spectrum is more diffuse and the peaks are shifted to longer wavelengths. Similar temperature-dependent emission spectra were observed for 9-VA and 9,10-DPA (fig. 3). These results are comparable to those found previously for substituted anthracenes [7–9]. As was done by these investigators, we too interpret the temperature-dependent spectral changes as the result of rotation of the naphthyl, phenyl or vinyl groups on the excited fluorophore. Presumably, this rotation permits increased conjugation between the molecular orbitals. This interpretation is supported by the work of Cherkasov [10], who demonstrated that such temperature-dependents shifts are only significant if the chemical structure

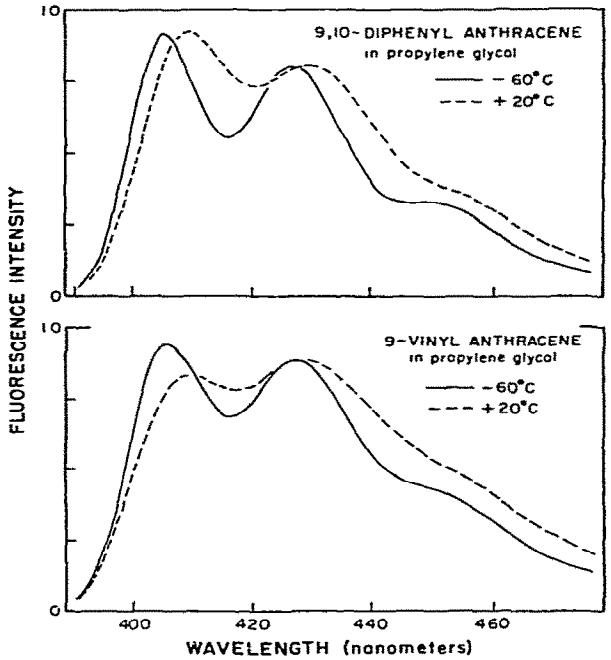

Fig. 3. Temperature-dependent emission spectra of DPA and 9-VA in propylene glycol.

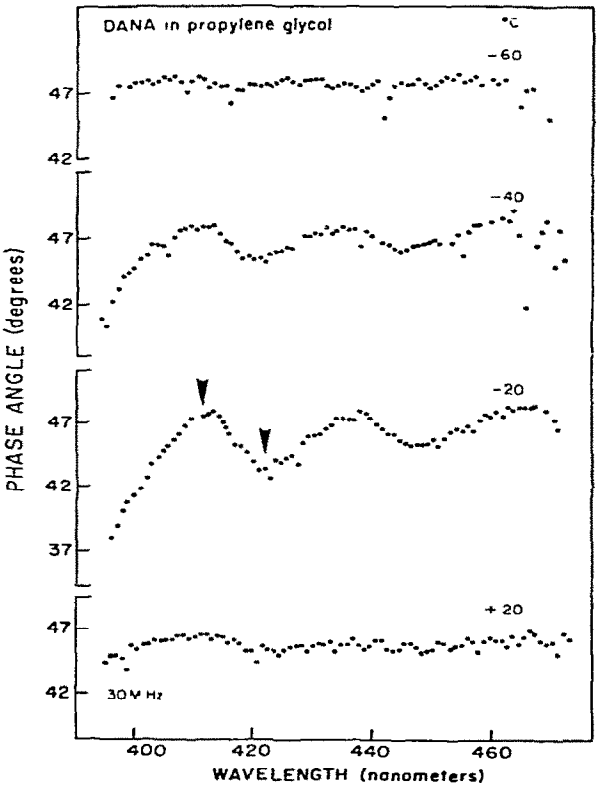

Fig. 4. Wavelength- and temperature-dependent phase angles of DANA in propylene glycol. The arrows indicate the wavelengths chosen to measure $\Delta\phi$.

permits electronic conjugation. The insertion of a methylene bridge between the anthracene moiety and the side chain decreased the temperature-dependent shifts to a much smaller value, comparable to methyl-substituted anthracenes.

The temperature-dependent spectra suggested that rotation of the side groups on the anthracene nucleus is a time-dependent process, which occurs during the lifetime of the excited state. Previously, we described in detail the phase and modulation properties of fluorophores which undergo excited-state reactions [11]. If a fluorophore undergoes an excited-state reaction to yield a relaxed state (R), then the phase angle of the R state is larger than that of the initially excited state (F). Figs. 2 and 3 indicate that the emission from that of the more coplanar fluorophores (R state) is red shifted from the initially excited noncoplanar molecules by about 10 nm. Consequently, the peak emissions from the 'coplanar' and 'noncoplanar' fluorophores are offset on the wavelength scale. Upon scanning the emission wavelength one alternatively selects, to a partial extent, the emission from the coplanar and the noncoplanar fluorophores. Since the coplanar state is thought to form from the initially excited state, one expects the emission from the coplanar state to be phase delayed relative to the F state.

The wavelength-dependent phase angles of DANA are shown in fig. 4. At the highest temperature (20°C), where the solvent is fluid, the corrected phase angles are essentially independent of wavelength. At this temperature the rate of naphthyl ring reorientation is faster than the rate of fluorescence decay, and hence emission is observed from a equilibrium distribution of excited-state conformations. As the temperature is decreased one notices 'sawtooth' pattern in the phase-angle spectra. The most dramatic phase spectra are seen near −20°C because the rotation is slowed by the increased viscosity, presumably at a rate comparable to that of the fluorescence life-

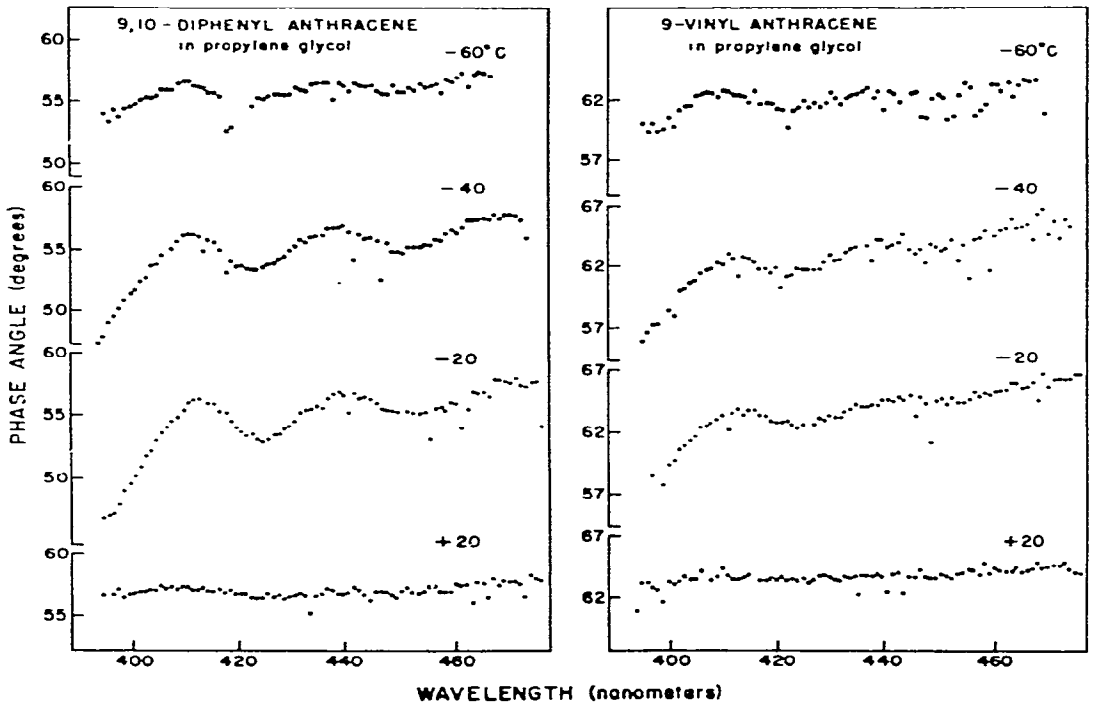

Fig. 5. Wavelength- and temperature-dependent phase angles of 9-VA and DPA in propylene glycol.

time. The sawtooth phase spectra decrease in amplitude at −40°C, and the sawtooth pattern is no longer detectable at −60°C. We attribute this decrease to inhibition of the naphthyl side rotation at higher viscosities (lower temperatures). Similar phase spectra were described previously for tolyl-substituted anthracenes [12].

Similar temperature-dependent phase spectra were seen for 9-VA and 9,10-DPA (fig. 5). Interestingly, our lowest available temperature (−60°C) was not adequate to inhibit completely the reorientation of the vinyl group of 9-VA or the phenyl groups of DPA. We attribute these differences to the size of the rotating units. The vinyl and phenyl groups are considerably smaller than the naphthyl groups on DANA, and these groups appear to rotate faster than the naphthyl groups at equivalent viscosities.

We obtained additional information on the temperature dependence of the reorientation rates by measuring the phase-angle difference between the peak (412 nm) and the minimum (422 nm) of the phase spectra (fig. 6). For each compound the phase difference shows a maximum at intermediate temperatures, and decreases at both higher and lower temperatures. As the size at the rotating unit increases, so does the temperature at which the phase difference is maximal.

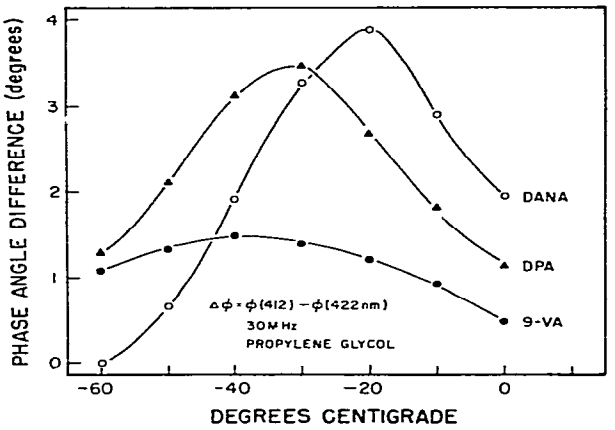

Fig. 6. Phase-angle difference between the shortest wavelength minimum and shortest wavelength maximum of DANA, DPA and 9-VA in propylene glycol. The phase-angle difference was obtained from $\Delta\phi = \phi(412) - \phi(422\ \text{nm})$.

4. Discussion

We demonstrated that rotational reorientation of side groups on anthracene could be detected from the fluorescence phase-angle spectra. This phenomenon is potentially useful for studies of the dynamic properties of proteins and membranes. Nonpolar molecules, such as those used in this study, bind readily to membranes, and probably also to proteins which contain hydrophobic sites. Furthermore, the rotational motions of the side groups are likely to be sensitive to the volume fluctuations of the surrounding macromolecule, just as these motions are sensitive to solvent viscosity. If the environment surrounding the fluorophore is very rigid or very fluid then the phase spectra will be independent of emission wavelength. Such a result is equivalent to an average decay time which is also independent of wavelength. Otherwise, the amplitude of the phase spectrum should provide an approximate indication of the motional freedom of the side groups. Comparison of fluorophores with side groups of varying sizes, and the temperature dependence of the phase spectra, should minimize the ambiguity resulting from the bell-shaped temperature dependence of the phase-angle spectra (fig. 6). We also note that the volume fluctuations needed for rotation of the phenyl and naphthyl moieties is probably comparable to the volume fluctuations expected for a protein, approx. 30 cm^3/mol [13]. Specifically, the molecular volumes of the phenyl and naphthyl groups are near 89 and 120 cm^3/mol. Rotation by 90° into the coplanar orientation is not likely to require the total volume, but rather the activation volume for the rotation is likely to be a fraction of this total. Hence, the time scale and volume requirements of these side group rotations are appropriate for studies of the volume fluctuations in proteins and membranes.

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