Solvent Relaxation of Prodan and Patman: A Useful Tool for the Determination of Polarity and Rigidity Changes in Membranes

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The time dependence of the solvent relaxation behavior of two polarity-sensitive dyes called Prodan and Patman has been investigated in detail in artificial membrane systems of different compositions. The works provides a comparison of the solvent relaxation behavior of the mentioned dyes, using steady-state and time-resolved fluorescence methods, and exemplifies their complementary use for membrane studies.

INTRODUCTION

Although the principles of solvent relaxation in fluorescence spectroscopy were discussed some decades ago, they have not been widely applied in biophysical studies. For example, blue shifts occurring in the fluorescence spectra of dyes when adsorbed to proteins have often been interpreted in terms of so-called hydrophobic or unpolar binding sites. However, this point of view completely neglects the possibility of a rather polar but rigid binding site which may strongly restrict the mobility of the dye environment, preventing solvent relaxation (and thus red-shifted emission) to occur on the fluorescence (nanosecond) time scale. The development of ultrafast spectroscopic methods with femtosecond resolution has led to an accurate determination of solvent relaxation in a large variety of polar solvents of low

viscosity and has allowed the physical interpretation within the framework of existing theoretical models. Only few fluorescence investigations of biomembrane structure, however, are based on this technique and exploit its advantages compared to other fluorescence methods used in membrane research, like quenching [1], energy transfer [2], lifetimes and lifetime distributions [3], excimer formation [4], and fluorescence anisotropy [3].

In this short review we want to illustrate the usefulness of the solvent relaxation method summarizing part of our recent work on the solvent relaxation behavior of the polarity-sensitive probes Prodan, 6-propanonyl-2-(dimethylamino)naphthalene, and Patman, 6-palmitoyl-2-[[trimethylammoniumethyl]methylamino]naphthalene chloride (for the structures; see Fig. 1). No attempt is made to cover all possible application in membrane studies; rather some examples are given on the kind of information available. Prodan and Patman proved to be especially useful probes for these studies, as both contain essentially the same fluorophore and show a very high polarity and time-dependent Stokes shifts (e.g., from 401 nm in cyclohexane to 531 nm in water) [5] but are located in somewhat different membrane regions as shown below.

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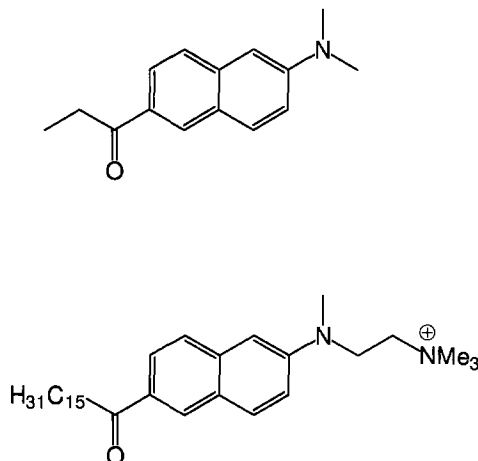[4] To whom correspondence should be addressed.

Fig. 1. Structure of Prodan (top) and Patman (bottom).

PRINCIPLES OF SOLVENT RELAXATION

For most chromophores electronic excitation results in an increase in the molecule's dipole moment and a changed electron distribution, disrupting the equilibrium between the solvated fluorophore and its environment in the electronic ground state. The dynamic process of establishing a new equilibrium in the excited state is called solvent relaxation (SR). The solvent relaxation process results in a continuous red shift of the probe's emission spectrum from the emission maximum frequency of the originally created Franck–Condon state [$\nu(0)$ for $t = 0$] to the emission maximum of a fully relaxed state ($\nu(\infty)$ for $t = \infty$), thereby called the R-state. Since a more polar solvent leads typically to a stronger stabilization of the polar R-state, the overall shift $\Delta\nu[\Delta\nu = \nu(0) - \nu(\infty)]$ increases with increasing solvent polarity. The fundamental "dielectric continuum solvation model" [6–8] predicts a linear relationship between $\Delta\nu$ and a dielectric measure of solvent polarity F for a large variety of solvents [9]. Although deviations from this linearity are found for a few aromatic solvents, it can be concluded for dielectric relaxation in phospholipid/water systems that changes in $\Delta\nu$ directly reflect polarity changes in the dye environment. The SR kinetics give information about the mobility of the dye environment. While the solvent response of low-viscosity solvents usually occurs on the femto- to picosecond time scale [9], it takes place on the nanosecond (ns) time scale if the dye is located in a viscous medium like phospholipid membranes [10].

The time evolution of the solvation energy relaxation process can be described by means of the normalized spectral response function or so-called "correlation function" $C(t)$:

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\Delta\nu} \qquad (1)$$

In all investigations on membrane labels done by our group employing a sub-ns "single-photon counting" apparatus [11–17], the solvent response appears to be rather complex and cannot be satisfactorily and consistently described by (multi)exponential relaxation models. Therefore, in order to characterize the overall time scale of the solvation response, we omit data fitting and use an (integral) average relaxation time:

$$\langle \tau_r \rangle \equiv \int\limits_0^\infty C(t)\ dt \qquad (2)$$

PRODAN AND PATMAN—A COMPARISON OF THEIR SOLVENT RELAXATION BEHAVIOR IN DMPC VESICLES

Prior to the description of the time-dependent relaxation behavior, it should be mentioned that some interesting information can already be extracted from the steady-state emission properties of Prodan and Patman in a simple model membrane system like DMPC (1,2-dimyristoylphosphatidylcholine) vesicles. It has been discussed whether the fluorescence behaviour of a polarity sensitive probe near a phase transition is better described by solvent relaxation processes or a probe relocation mechanism [18–20]. The steady-state emission spectra of Prodan in DMPC vesicles are broader and red-shifted compared to Patman, suggesting a more polar and heterogeneous environment of the Prodan chromophore [18]. While the spectra of Patman are quite similar in small unilamellar (SUV) and multilamellar vesicles (MLV), Prodan is able to distinguish between both types of vesicles for temperatures below the phase transition temperature T_c, showing different widths of the emission spectra. A more heterogeneous environment of Prodan in SUV compared to MLV, most probably due to the high intrinsic curvature of the SUV facilitation water penetration, has been inferred.

The ability to differentiate between SUV and MLV clearly seems to be associated with the length of the acyl chain: while the behavior of Prodan is strikingly different in both types of vesicle systems, only minor differences were observed with Patman.

The observation of red-shifted emission spectra with increasing excitation wavelength λ_{ex} as well as the decrease in steady-state anisotropies (r_{ss}) with increasing emission wavelength λ_{em} [13] can be used as first and
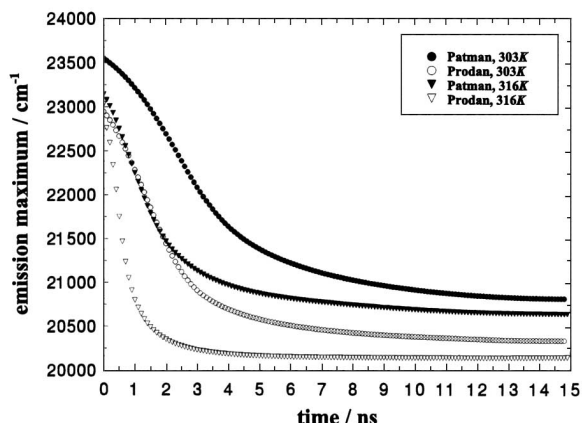
Fig. 2. Time-resolved emission maxima (cm⁻¹) at different temperatures for Prodan and Patman as a function of time (ns).

Table I. Mean Relaxation Times $\langle\tau_r\rangle$ (ns) and Stokes' Shift (nm) at Different Temperatures (K) as Calculated According to Eq. (2) for Patman in DPPC and DHPC SUV

	Temperature		
	293	313	325
	DPPC		
$\langle\tau_r\rangle$	n.d.[a]	3.7	1.2
Shift	5	17	49
	DHPC		
$\langle\tau_r\rangle$	2.6	2.0	<0.7
Shift	31	43	>31

[a] Not determined due to a small shift.

simple indications for the occurrence of solvent relaxation. A decrease in r_{ss} with increasing λ_{em} is caused by the selection of longer-living, relaxed fluorophores, which have more time to reorient.

Fluorescence decays in DMPC vesicles can be well described using a biexponential model for both dyes at all emission wavelengths. For $\lambda_{em} \geq 470$ nm one decay component is always obtained with a negative preexponential factor. An increase in the mean decay times is observed with increasing λ_{em}, as expected for increasing contributions of relaxed states.

TRES (time-resolved emission spectra) and correlation functions were calculated for both Patman and Prodan in SUV composed of DMPC at different temperatures above the phase transition temperature. The TRES of Patman are generally blue-shifted (~15 nm) compared with those of Prodan, while the solvent response of Patman is about twofold slower than for Prodan (see Fig. 2, which shows the time evolution of the

emission spectra). For example, average relaxation times are 2.2 and 3.5 ns at 30°C and at 0.7 and 1.8 ns at 43°C for Patman and Prodan, respectively. These results suggest that the chromophore of Patman is localized in a less polar and/or more restrictive environment compared to Prodan. This view has also been supported by high-resolution ¹H NMR results [13].

APPLICATIONS: SOLVENT RELAXATION BEHAVIOR OF PRODAN AND PATMAN IN DIFFERENT MEMBRANE SYSTEMS

Substitution of Diacyl- by Diether Lipids

The SR of Prodan and Patman has been used to characterize the headgroup mobilities of vesicles containing monoether lipids [21,22] as well as asymmetric [16] and symmetric [17] diether lipids. For the latter class comparative studies were done between the diether- and the corresponding diacyl lipid, i.e., the solvent relaxation was studied in 1,2-dipalmitoyl-phosphatidylcholine (DPPC) versus 1,2-dihexadecyl-phosphatidylcholine (DHPC) [17] and in 1-stearoyl-2-lauroylphosphatidylcholine versus 1-O-stearyl-2-O-laurylphosphatidylcholine [16].

In summary, it was concluded that the substitution of the acyl- by ether-linkages leads to considerably faster SR as monitored by both fluorophores. As an example, the SR characteristics of Patman in SUV composed of the symmetric diacyl lipid (DPPC) and diether lipid (DHPC) are given in Table I [17]. The $\Delta\nu$ dependence observed for these systems gives further direct evidence that the ether-linkage allows more efficient water penetration in the glycerol region.

Content of Negatively Charged Lipids in the Absence and Presence of Calcium

Biological membranes contain only relatively small amounts of anionic phospholipids, nevertheless, their presence is essential for many physiological processes on the cellular level. Thus, the labels Prodan and Patman have been used to study the influence of phosphatidylserine (PS) and calcium ions on the phospholipid headgroup mobility in PS/PC-SUV [14]. Patman monitors a slight deceleration of the headgroup mobility with increasing PS content in the absence of Ca^{2+} (see Table II), suggesting a lower flexibility of the PS headgroup. In contrast to pure PC vesicles, the addition of 3 mM Ca^{2+} leads to a considerable deceleration of the solvent relaxation in PS-containing vesicles as observed with

Table II. Mean Relaxation Times $\langle \tau_r \rangle$ (ns) for Patman and Prodan in PC/PS Vesicles in the Absence and Presence of 3 mM Ca^{2+}

		$\langle \tau_r \rangle$ at molar fractions PS
		0	10	20	30	40	50
Prodan	Absent Ca^{2+}	1.0	1.1	1.1	1.1	1.2	1.0
	With Ca^{2+}	1.1	1.1	1.2	1.3	1.3	1.5
Patman	Absent Ca^{2+}	2.1	2.0	2.0	2.2	2.3	2.5
	With Ca^{2+}	2.0	2.0	2.3	2.6	2.8	3.5

both dyes (see Table II). This effect increases with increasing PS content. It was shown that the deceleration of the headgroup mobility with increasing PS content is much more pronounced when Ca^{2+} is present. These results indicate a tighter phospholipid headgroup packing with increasing PS content and suggest a bridging of PS molecules by Ca^{2+} within the plane of the membrane, leading to a decrease in lipid mobility.

Binding of Vitamin K-Dependent Proteins

Although the molecular mechanism of the interaction of the vitamin K-dependent protein prothrombin with negatively charged membrane surfaces has already been investigated using fluorescence spectroscopy [23,24], a clear mechanistic picture is still missing. As binding of this class of calcium-dependent proteins should affect mainly the lipid headgroup fluorescence techniques like pyrene excimer [23] and DPH anisotropy [24], preferentially probing the hydrophobic backbone might not be sensitive enough to detect protein-induced changes in the membrane headgroup organisation.

Recently, we have compared protein-induced changes in the SR kinetics monitored by Patman and Prodan with changes in the DPH steady-state anisotropy for the binding of prothrombin to PS-containing membranes [14,25]. Using apparent saturation protein concentrations [26] the SR times $\langle \tau_r \rangle$ for Prodan and Patman in SUV composed of PC/PS 80:20 increased by approximately 100 and 30%, respectively. On the other hand, the observed increase in membrane order under similar conditions reported by DPH anisotropy is comparatively small ($<$ 5%) [25]. Thus, the binding of this peripheral protein considerably rigidifies the phospholipid headgroup region but leads only to a very small increase in the packing density of the hydrocarbon region of the bilayer. The observed deceleration of the SR process is larger for Prodan, which is bound near the surface of the membrane, than for Patman, which is located closer to the hydrocarbon region. Apparently, binding of these proteins affects predominantly the outermost region of

the membrane, where the amino and the carboxyl groups of the serine headgroups are supposed to be located. These observations help to draw conclusions on the molecular mechanism for the prothrombin membrane interaction and illustrate the high sensitivity of the SR method for the binding of peripheral membrane proteins.

SUMMARY

Studying the time-dependent SR behavior of polarity-sensitive probes like Prodan and Patman has been shown to be a versatile tool for the investigation of membrane dynamics. These two dyes are especially useful, allowing the monitoring of different membrane regions with essentially the same chromophore. The approach has been extended to other membrane probes like the set of n-anthroyloxy fatty acids [15], which can be used to probe the relaxation behavior in different depths of the bilayer.

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