Fluorescence and Absorption Spectroscopic Properties of Dipyrromethene boron Difluoride (BODIPY) Derivatives in Liquids, Lipid Membranes, and Proteins

Jan Karolin,† Lennart B.-A. Johansson,*,† Leif Strandberg,‡ and Tor Ny‡

Contribution from the Department of Physical Chemistry, University of Umeå, S-901 87 Umeå, Sweden, and Department of Medical Biochemistry and Biophysics, University of Umeå, S-901 87 Umeå, Sweden

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Abstract: Light spectroscopic properties of a recently developed fluorophore, 4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene (BODIPY) and its derivatives, have been studied in different solvents, lipid membranes (modeled by lyotropic lamellar and cubic liquid crystals) as well as a protein. The absorption spectrum shows a strong {$\epsilon \approx 90\,000$ M^{-1} cm^{-1}} $S_0 \rightarrow S_1$ transition at about 500 nm and a higher $S_0 \rightarrow S_2$ transition at about 375 nm, which is 20 times weaker. The electronic dipole of the $S_0 \rightarrow S_1$ transition is polarized along the long axis of the chromophore, as shown by linear dichroism spectroscopy (LD). The $S_0 \rightarrow S_2$ band contains a mixture of, presumably, in-plane-polarized transitions. For the $S_0 \leftrightarrow S_1$ transitions, the limiting fluorescence anisotropy, $r_0 = 0.37$. The fluorescence quantum yields are typically higher than 0.8. The calculated radiative lifetime of $\tau_0 = 5.6$ ns in methanol is in reasonble agreement with the experimental value of $\tau_0 = 5.9 \pm 0.2$ ns. An efficient overlapping between the strong $S_0 \leftrightarrow S_1$ transitions yields a Förster radius of $R_0 = 57 \pm 1$ Å. The fluorescence lifetime and spectra are pH independent and change little with solvent polarity. The influence of oxygen quenching is typically less than 5% in liquids. The fluorescence of BODIPY is quenched by Trp ($K = 15$ M^{-1}) and Tyr ($K = 14$ M^{-1}). The quenching by Phe is less efficient, at least by one order of magnitude. The fluorescence relaxation of BODIPY is monoexponential when solubilized in lipid vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and a cubic liquid–crystalline phase of monoolein. The fluorescence decay of BODIPY, when covalently bound to the cystein residue of a mutant form of the plasminogen activator inhibitor 1 (PAI-1) is very well described by a sum of two exponential functions. The major component contributes with *ca.* 95% to the total intensity. In one of the PAI-1 mutants studied here, a Cys replaces the reactive center Ser 344 of the wildtype PAI-1. From the fluorescence anisotropy we conclude that the rotational correlation function of BODIPY in PAI-1 contains rapid motions (unresolved on the sub-ns time scales) and (at least) two rotational correlation times of about 3 and 50 ns. The longest correlation time is compatible with the tumbling motion of the protein molecule in water. Furthermore, it appears that the local rotational mobility of the BODIPY moiety in the reactive center of PAI-1 is restricted. The orientation and the rotational correlation function of different lipid derivatives of BODIPY in various lipid bilayers were studied by LD and time-resolved fluorescence spectroscopy.

Extrinsic fluorophores may serve as important reporter molecules of dynamics, structure, and function of lipid membranes, peptides, and proteins. The fluorescence experiment reveals direct information about the probe molecules but informs only indirectly about the particular system where the probes reside. This is the major drawback of any probe technique. However, by a critical choice of probes, regarding a specific question, it could still be possible to extract important molecular information about the system. For example, probe molecules whose photophysical and spectral properties depend strongly on the local polarity are not suited for fluorescence depolarization experiments, where an analysis in terms of molecular motions and/or molecular separation is desirable. Instead such probes are better suited for estimations of local polarity. For fluorescence depolarization studies, it is desirable to work with chromophores having strong electronic transitions and whose electronic transition dipoles are well-defined in the spectra as well as regarding their polarization in a molecular frame. The quantum yield of fluorescence should be greater than 0.5, perferably 1.0. It is also desirable to use relatively small fluorescent groups, in order to avoid perturbations of the biological activity. Moreover, the spectral overlap with intrinsic chromophores, such as aromatic amino acids, phosphates, carbonyl groups, etc., should be avoided, and consequently we must use excitation and emission wavelengths greater than about 350 nm. Moreover, the fluorophores should be pH independent and possess high symmetry ($>C_{2v}$ and ideally $D_{\infty h}$). Taken together, we realize that very few, if any fluorophore, will meet all of these criteria.

In the present article we report on the absorption and fluorescence spectroscopic properties of the chromophore, 4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene (BODIPY) which is potentially an interesting candidate that meets most of the criteria mentioned above. This work also aims at briefly characterizing the behavior of specifically designed BODIPY probes in lipid bilayers and proteins. Historically, BODIPY was synthesized for the first time by Treibs and Kreuzer,[1] in 1968. Latter an ionic derivative was synthesized by Worries,[2] and several reactive forms have recently been prepared by Kang and Haugland.[3] Different derivatives of BODIPY have been prepared for lipid and protein systems and some of them are displayed in Figure 1. We have examined 3,5-didecyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene ($C_{10}BDYC_{10}$) and 5-decyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indazene-3-propionic acid ($C_{10}BDYC_3$) when solubilized in lipid model membranes. For this, we use lamellar liquid crystals of monoolein and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and a cubic liquid–crystalline phase of monoolein. A cystein specific derivative of iodoacetamide [*N*-(4,4-difluoro-5,7-dimethyl-4-borata-3a-azonia-

† Department of Physical Chemistry.
‡ Department of Cell and Molecular Biology.
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(1) Treibs, A.; Kreuzer, F.-H. *Liebigs Annalen Chem.* **1968**, *718*, 203.
(2) Wories, H. J.; Koek, J. H.; Lodder, G.; Lugtenberg, J.; Fokkens, R.; Driessen, O.; Mohn, G. R. *Recl. Trav. Chim. Pays-Bas* **1985**, *104*, 288.
(3) Haugland, R. P.; Kang, H. C. *Chemically Reactive dipyrrometeneboron Difluoride Dyes*; U.S. Patent 4, *774*, 339, 1988.

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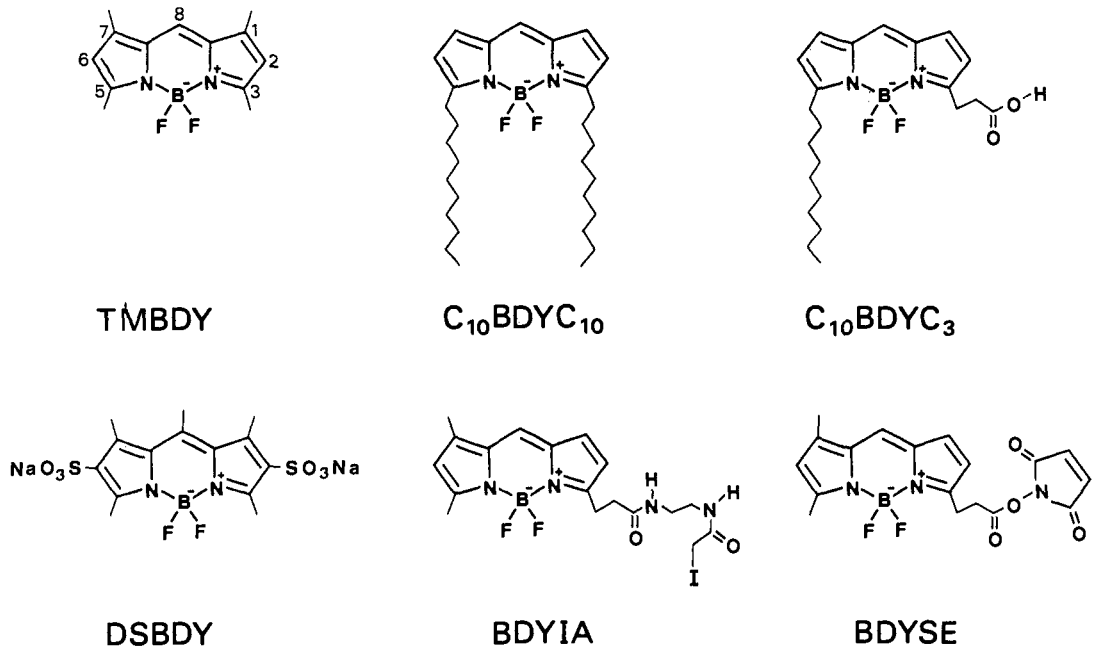

Figure 1. Structure formulas and acronyms of the different BODIPY derivatives studied in this work. The chemical names are given in the section of Materials and Methods.

4a-aza-*s*-indacene-3-propionyl)-*N'*-(iodoacetyl)ethylenediamine (BDYIA)] has been used to label the Ser 344 → Cys mutant of PAI-1 (the Plasminogen Activator Inhibitor 1).[4] The fluorescence spectroscopic properties of BODIPY labeled mutant were characterized, while the biological activity of this and other labeled mutants of PAI-1 will be the subject of a future paper (Strandberg, L. et al. accepted for publication in *Thromb. Res.*). The rather hydrophobic BODIPY derivative, 3,3',4,4-difluoro-1,3,5,7-tetramethyl-4-borata-3a-azonia-4a-aza-*s*-indacene (TMBDY), and the water soluble 4,4-difluoro-1,3,5,7,8-pentamethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-2,6-disulfonic acid, disodium salt (DSBDY) were examined in liquids, as well as in polymer films.

Materials and Methods

The followinng BODIPY derivatives, all of which are commercially available from Molecular Probes, Inc., Eugene, OR (USA), were used: 3,5-didecyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene (C_{10}BDYC_{10}), 5-decyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indazene-3-propionic acid (C_{10}BDYC_3), 4,4-difluoro-1,3,5,7,8-tetramethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-2,6-disulfonic acid (DSBDY), *N*-(4,4-difluoro-5,7-dimethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-3-propionyl)-*N'*-(iodoacetyl)ethylenediamine (BDYIA), and 5-decyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene-3-propionic acid, succimidyl ester.

Monoolein was purchased from Sigma (USA), and it was used without further purification. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (USA). The purity of the lipid was better than 99% as checked by thin-layer chromatography at our laboratory.

Vesicles were prepared by sonication according to the following procedure. Appropriate amounts of the dry powder of the lipid was dissolved in a mixture of chloroform/methanol (2:1 vol/vol). The solvent was evaporated, and the samples were dried at 320 K and 0.1 Torr during at least 2 h. Afterwards, 3 mL of water was added, and the suspension was freezed with liquid nitrogen and thawed ten times. The samples were then sonicated eight times in intervals of 5 min. During the sonication the sample was cooled at about 283 K. The sonicator was a Soniprep 150 (MSE Scientific Instruments, England) supplemented with an exponential microprobe. The level of the amplitude used as 10–14 μm.

Cubic and lamellar liquid crystals were prepared by dissolving the lipid and the fluorophore in a mixture of chloroform/methanol (2:1 vol/vol). The solvent was evaporated, and the samples were dried at 320 K and 0.1 Torr during at least 2 h. Weighed amounts of water were added, the ampule (or cuvette) was sealed, and the sample was equilibrated.

Polyvinylalcohol films were prepared by dissolving 5 g of PVA powder in 50 mL of water/methanol (70:30%, vol/vol). The solution was heated to 70 °C, stirred during a 10-min time interval, and slowly cooled to room temperature. The probe molecules were added from a stock solution and after homogenous mixing the polymer solution was spread on a clean glass plate. The films could be stretched *ca.* six times after 24 h.

The Ser 344 → Cys mutant of PAI-1 was constructed by oligonucleotide directed mutagenesis, essentially as described in ref 5. This mutation introduces a unique Cys at the P3 position of the reactive center of PAI-1, without any major effects on protein structure and function (data not shown). The mutant protein was produced in *Escherichia coli* and purified to homogeneity essentially.[5,6] The labeling of the mutant PAI-1 with the BODIPY in 50 mM sodium phosphate (pH = 6.8), 150 mM NaCl, 0.01% Tween 80, 3% DMSO, and 1 mM TCEP was performed in the dark for 18 h at 277 K. The PAI-1 and BODIPY concentrations were typically 10 and 200 μM, respectively. After the labeling reaction, excess of BODIPY was removed by gel filtration on a Sephadex G25 column.

The steady-state fluorescence spectra and anisotropies were obtained using a SPEX Fluorolog 112 instrument (SPEX Ind., NJ, USA), equipped with Glan–Thompson polarizers. The spectral bandwidths were 5.6 and 2.7 nm for the excitation and emission monochromators, respectively. The fluorescence spectra were corrected. The fluorimeter was calibrated by using a standard lamp from the Swedish National Testing and Research Institute, Borås.

A PRA 3000 system (Photophysical Research Ass. Inc., Canada) was used for single-photon-counting measurements of the fluorescence decay. The excitation source is a thyratron-gated flash lamp (Model 510C, PRA) filled with deuterium gas and operated at about 30 kHz. The excitation wavelengths were selected by interference filters (Omega/Saven AB, Sweden) centered at 470 nm (HBW = 9.3 nm). The fluorescence emission was observed above 520 nm through a long pass filter Schott KV 520 (Schott, West Germany). The maximum absorbance of all samples was kept below 0.08 which corresponds to a total concentration of less than 10^{-6} M. The half width of the instrumental response time was between 1.5 and 2 ns. The time-resolved polarized fluorescence decay curves were measured by repeated collection of photons during 200 s, for each setting of the polarizers. The emission polarizer was fixed, and the excitation polarizer rotated periodically. In each experiment the decay curves $F_{\parallel}(t)$ and $F_{\perp}(t)$ were collected. The subscripts $\parallel$ and $\perp$ refer to an orientation of the emission polarizer parallel and are perpendicular with respect to the excitation polarizer. From these a sum curve

(4) Hekman, C. M.; Loskutoff, D. J. *J. Biol. Chem.* **1985**, *260*, 11581. Loskutoff, D. J.; Sawdey, M.; Mimuro, *J. Prog. Haemostasis Tromb.* **1988**, *9*, 87.

(5) Strandberg, L.; Lawrence, D. A.; Johansson, L. B.-A.; Ny, T. *J. Biol. Chem.* **1991**, *266*, 13857.

(6) Strandberg, L.; Erickson, Ny, T. *J. Biol. Chem.* **1990**, *265*, 20293.

$$S(t) = F_{\parallel}(t) + 2GF_{\perp}(t)$$

and a difference curve

$$D(t) = F_{\parallel}(t) - GF_{\perp}(t)$$

were calculated. The correction factor, G, was obtained by normalizing the total number of counts $F_{\parallel}$ and $F_{\perp}$ collected in $F_{\parallel}(t)$ and $F_{\perp}(t)$, respectively, to the steady-state anisotropy, r_s, as

$$G = (1 - r_s)(1 + 2r_s)^{-1}F_{\parallel}(F_{\perp})^{-1}$$

The fluorescence decay curves were deconvoluted on a IBM compatible PC, by using a nonlinear least-square analysis based on the Levenberg–Marquardt algorithm.

Linear dichroism (LD) spectra were recorded on a JASCO J-720 supplemented with an Oxley device and the absorption spectra on a Cary 119 spectrophotometer. Details of studying macroscopically aligned lamellar liquid crystals, and the interpretation of data are given elsewhere.[7]

Absorption spectra were recorded on a Cary 219 (Varian, USA) spectrophotometer supplemented with sheet type polarizers (HNB'P Polarizer, U.K.).

The Förster radius (R_0) of donor–donor transfer was determined in the following way. The corrected fluorescence spectrum, $F(\nu)$, and the molar absorptivity, $\epsilon(\nu)$ were determined. These were used to calculate the Förster radius from

$$R_{0f} = \left[\frac{9000(\ln 10)\langle\kappa^2\rangle\Phi I\nu}{128\pi^5 n^4 N_A}\right]^{1/6}$$

$$I\nu = \int \epsilon(\nu) f(\nu)\nu^{-4}\, d\nu$$

$$f(\nu) = \frac{F(\nu)}{\int F(\nu)\, d\nu}$$

Here ν, N_A, n, and Φ denote the wavenumber of light, Avogadro's constant, the refractive index of the medium, and the fluorescence quantum yield, respectively.[8] In the equation $\langle\kappa^2\rangle$ is the mean value of the orientational part of a dipole–dipole interaction. In order to calculate the Förster radius it is convenient to choose $\langle\kappa^2\rangle = 2/3$, as a reference state. This average value is relevant in a three-dimensional system for the interaction between rapidly rotating dipoles and is often referred to as the dynamic limit or fast case. We denote this Förster radius by R_0.

The fluorescence quantum yield, Φ, was determined as follows. Fluorescein in water was used as a standard with a reported[9] quantum yield of 0.93. The quantum yield was calculated from

$$\Phi = \Phi_{ref}\frac{F_{ref}}{F}\frac{[1 - \exp(-A_{ref}\ln 10)]n^2}{[1 - \exp(-A\ln 10)]n_{ref}^2}$$

Here, F denotes the integral of the corrected fluorescence spectrum, and A is the absorbance at the excitation wavelength.

Basic Theory

Linear Dichroism. Some lyotropic lamellar liquid crystals align spontanously between quartz slides and form *macroscopically* uniaxial systems with the optic axis being perpendicular to the bilayers. The orientation of chromophoric molecules solubilized in such systems is uniaxially anisotropic. If linearly polarized light propagates at an angle of tilt to the optic axis, its absorption will depend on the direction of the polarization plane (as is illustrated in Figure 2). The difference in absorption of light polarized in and out of the plane of the bilayer defines the linear dichroism, LD. LD yields information about the average orientation of the electronic transition dipole moment of the chromophore in terms of an order parameter;

$$S = \int_0^{\pi} f(\beta)\frac{1}{2}(3\cos^2\beta - 1)\sin\beta\, d\beta \qquad (1)$$

Here β is the angle between the electronic transition dipole moment and the normal to the lipid bilayer and $f(\beta)$ is the normalized orientational distribution function. The order parameter can take values $-1/2 \le S \le 1$, where the limits correspond to a perfect orientation perpendicular and parallel to the normal of the bilayer, respectively. In particular, $S = 0$, for an isotropic orientational distribution.

Fluorescence Anisotropy. The orientational correlation function of the electronically excited molecules in a macroscopically isotropic system is described conveniently by the time-resolved fluorescence anisotropy,

$$r(t) = \frac{F_{zz}(t) - F_{zx}(t)}{F_{zz}(t) + 2F_{zx}(t)} \qquad (2)$$

$F_{zz}(t)$ and $F_{zx}(t)$ denote the fluorescence emission as Z-polarized excitation light propagates along the laboratory X-axis, while the emission is monitored along the Y-axis with the polarizer settings being parallel to the Z and X axes, respectively. It can be shown that $r(t)$ depends *only* on the orientational dynamics of the molecules, if the excited state processes are independent of its orientational dynamics (see, for example, ref 10 and papers cited therein) and provided energy migration is negligible. Within these assumptions one obtains that

$$r(t) = \frac{2}{5}\sum_{m=-2}^{2}\langle D_{m0}^{(2)}(\Omega_{ML}^0)D_{m0}^{(2)*}(\Omega_{M'L})\rangle \qquad (3)$$

which is the orientational correlation function written on the basis of second rank irreducible Wigner rotational matrices.[11] The orientation of the molecules at the time of excitation (*i.e.*, $t = 0$), and at a time t later, is described by the eulerian angles of Ω_{ML} and $\Omega_{M'L}$, respectively. The subscripts indicate that the transformations are from the molecule fixed (M, M') frame to a laboratory fixed (L) frame. The initial anisotropy is $r(0) = 2/5$, if the absorption and emission transition dipoles are parallel.

Consider a fluorescent molecule covalently linked to a spherical protein molecule, and assume that the local mobility of the fluorophore is independent of the overall rotation of the protein. For this case eq 3 can be rewritten as

$$r(t) = \frac{2}{5}\exp(-t/\phi_c)\sum_{m=-2}^{2}\langle D_{m0}^{(2)}(\Omega_{MD}^0)D_{m0}^{(2)*}(\Omega_{M'D})\rangle \qquad (4)$$

Here ϕ_c is the rotational correlation time of the protein, and the expression within the bracket represents the *local* rotational correlation function of the fluorophore. If the rotational motion of the protein is diffusive, then the correlation time see, e.g. ref 12

$$\phi_c = \frac{\eta V_h}{kT} \qquad (5)$$

where η is the solvent viscosity, V_h is the hydrodynamic volume, and kT has its usual meaning. The evaluation of the correlation function of eq 4 requires a physical model that accounts for the restricted mobility of the probe at its site, a task which is far from trivial. We will assume here that the local correlation, $\rho(t)$, function can be written as

(7) Johansson, L. B.-A.; Davidsson, A. *J. Chem., Soc. Faraday Trans. I* **1985**, *81*, 1373.
(8) Kawski, A. *Photochem. Photobiol.* **1983**, *38*, 487.
(9) Weber, G.; Teale, F. W. J. *Trans. Faraday Soc.* **1958**, *54*, 640.
(10) Szabo, A. *J. Chem. Phys.* **1984**, *81*, 150.
(11) Brink, D. M.; Satchler, G. R. *Angular Momentum*; Oxford University Press: Oxford, 1968.
(12) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry*, Part II; H. Freeman: San Francisco, 1980.

$$\rho(t) = \sum_k \rho_k(t) \exp(-t/\Phi_k) + \rho_\infty \quad (6)$$

In eq 6 the Φ_k's are the effective rotational correlation times. The constant value of $(2/5)\rho_\infty$ corresponds to the residual fluorescence anisotropy, reached at sufficiently long times $t \geq t_\infty$. The residual values of ρ_∞ are restricted by $0 \leq \rho_\infty \leq 1$, depending on the orientational distribution in the binding site. In particular, if the orientational distribution of the fluorophore is uniaxial about the Z_D axis of a local frame, as is the case in a membranes (*e.g.*, lipid vesicles), then the residual value, ρ_∞, is given by

$$\rho(t_\infty) = S^2 \quad (7)$$

In eq 7, it is assumed that the orientational distributions of ground and excited states are the same.

Results

BODIPY in Liquids and PVA. *The absorption spectrum* of BODIPY (see Figure 3) shows a strong $S_0 \rightarrow S_1$ transition (for example; ϵ (503.5 nm) = 88 700 M^{-1} cm^{-1} for TMBDY in ethanol) located at about 500 nm and a weak broad $S_0 \rightarrow S_2$ transition located at about 375 nm. Actually, if the BF_2 atoms are removed from BODIPY and the conjugated systems are turned to the *all-trans* conformation the chromophore is converted into a cyanine dye having[13] a strong ($\epsilon \approx 100\,000$ M^{-1} cm^{-1}) electronic transitions at about 600 nm and a much weaker ($\epsilon \approx 2000$ M^{-1} cm^{-1}) at about 400 nm. Cyanine dyes undergo *trans–cis* isomerizations upon electronic excitation and are therefore not suitable as bioprobes in fluorescence depolarization studies. The BODIPY molecules used in this work can be considered as a stabilized cyanine dye.

The *linear dichroism (LD) spectra* were recorded for TMBDY (3,3′,4,4-difluoro-1,3,5,7-tetramethyl-4-borata-3a-azonia-4a-aza-*s*-indacene) and DSBDY (4,4-difluoro-1,3,5,7,8-pentamethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-2,6-disulfonic acid, disodium salt) dissolved in stretched PVA (polyvinylalcohol) films. In these experiments, $LD(\lambda) \equiv A_Z(\lambda) - A_Y(\lambda)$ was determined with the direction of stretching being parallel to the *Z*-axis of a laboratory fixed frame. For the $S_0 \rightarrow S_1$ bands of TMBDY and DSBDY we obtain the reduced linear dichroisms of $LD_r(\lambda) \approx 0.5$ and $LD_r(\lambda) \approx 1.5$, respectively. This shows that the electronic transition dipole tends to orient parallel with the *Z*-axis. Elongated molecules orient with their long axis preferentially parallel to the stretching direction.[14] Therefore, the present data strongly suggest that the $S_0 \rightarrow S_1$ transition is polarized parallel to the long axis of the BODIPY. The weak and broad $S_0 \rightarrow S_2$ transition located at about 375 nm yields positive but lower LD_r values, which shows that the transition dipole can neither be polarized parallel to the molecular C_2 axis nor parallel to the long axis.

The *fluorescence spectrum* of the BODIPY is displayed in Figure 3 and reveals a strong overlapping with the absorption spectrum. These spectra, recorded for 3-(5,7-dimethyl BODIPY)-1-propionic acid, succimidyl ester in propanediol (at 237 K), are typical for different BODIPY derivatives in solvents. The spectral shifts are usually within a few nanometers. The efficient overlapping between the $S_0 \leftrightarrow S_1$ transitions yields a high value on the Förster radius R_0. For TMBDY in ethanol we obtain that $R_0 = 57 \pm 1$ Å. The quantum yields of fluorescence, $\Phi > 0.8$ in the different organic liquids (acetone, acetonitrile, ethanol, methanol) and solutions, are studied here. These results are in good agreement with the values previously reported by Johnson et al.[15]

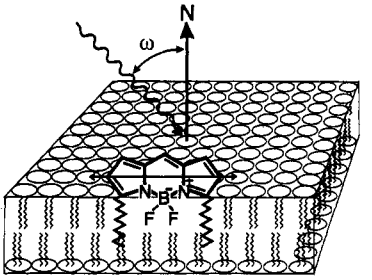

Figure 2. A schematic illustration of the orientation of $C_{10}BDYC_{10}$ in lipid bilayers of DOPC. The normal to the membrane is indicated by the vector *N*, while the arrows in the plane of the BODIPY chromophore represent the directions of the $S_0 \leftrightarrow S_1$ electronic transition dipole moments. In the linear dichroism (LD) experiments polarized light impinges at an angle (ω) of usually 45° to *N*. For further details see ref 7.

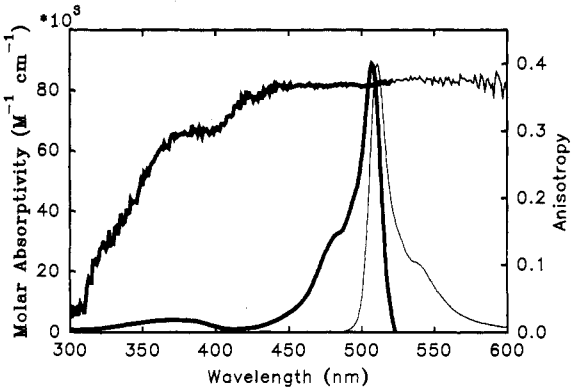

Figure 3. Absorption (—) and corrected fluorescence (—) spectra of BDYSE (4,4-difluoro-5,7-dimethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-1-propionic acid, succimidyl ester). The fluorescence excitation (**bold noisy**) and emission anisotropies in propanediol at 237 K are also displayed.

The *time-resolved fluorescence* experiments give typically fluorescence lifetimes of $\tau \approx 6$ ns. In Table 1, we summarize the results obtained for different BODIPY derivatives in various systems. The influence of polarity on the fluorescence lifetime is small, as judged from the values found for TMBDY in various solvents. By using the fluorescence lifetime and quantum yield one can calculate the radiative lifetime τ_0. For TMBDY in methanol, we obtain that $\tau_0 = 5.9 \pm 0.2$ ns from the experimental values of $\tau = 5.5$ ns and $\Phi = 0.93 \pm 0.03$. This result agrees reasonably well with the calculated value of $\tau_0 = 5.6$ ns, predicted by the modified Strickler–Berg equation.[16]

The *fluorescence quenching* by oxygen is less than *ca.* 5% in solvents, as could be seen from Table 1. The quenching of the BODIPY by the aromatic amino acids was examined. For this TMBDY was dissolved in a mixture of water with DMSO, and the fluorescence intensities (*F*) were monitored as a function of concentration of Trp, Tyr, and Phe at pH ≈ 12. The high pH and the solvent mixture were needed for obtaining concentrations high enough to quench BODIPY. Under these conditions Tyr appears as the tyrosinate anion, Tyr^-. The results are presented as Stern–Volmer graphs in Figure 4. Clearly, Trp and Tyr^- are as efficient quenchers of the BODIPY, while Phe is at least ten times less efficient, as could be seen from the figure. The fluorescence lifetimes, determined from single photon counting experiments (*cf.* Table 1), show that Trp and Tyr^- not only shorten the lifetime of TMBDY, but also give rise to a biexponential fluorescence relaxation. Furthermore, the relative decrease of the average fluorescence lifetime, upon adding Trp, Tyr^-, and Phe agrees well, with the observed decrease of the fluorescence intensity (F_0/F). These results are compatible with dynamic quenching mechanisms.

(13) Malhotra, S. S.; Whiting, M. C. *J. Chem. Soc.* **1960**, *Part IIIp*, 3812.
(14) Michl, J.; Thulstrup, E. W. *Spectroscopy with Polarized Light*; VCH Publishers, Inc.: New York, 1986.
(15) Johnson, I. A.; Kang, H. C.; Haugland, R. P. *Anal. Biochem.* **1991**, *198*, 228.
(16) Birks, J. B. *Photophysics of Aromatic Molecules*; Wiley-Interscience: London, 1970.

Table 1. Fluorescence Lifetimes (τ_1 and τ_2) of Different BODIPY Derivatives in Liquids, Liquid Crystals, and a Protein[a]

fluorophore	medium	τ_1 (ns)	τ_2 (ns)
TMBDY	water	5.80	
TMBDY	ethanol	5.45	
TMBDY	ethanol:acetone (67:33)	5.38	
TMBDY	ethanol:acetone (33:67)	5.46	
TMBDY	acetone	5.29	
TMBDY	acetonitrile	5.42	
TMBDY	methanol	5.53	
TMBDY	methanol (degassed)	5.82	
TMBDY	acetonitrile:methanol (33:67)	5.49	
TMBDY	acetonitrile:methanol (67:33)	5.47	
TMBDY	DMSO:water (20:80) pH ≈ 12	5.8	
TMBDY	DMSO:water (20:80) 91 mM Trp, pH ≈ 12	3.0 (90%)	1.3
TMBDY	DMSO:water (20:80) 48 mM Tyr, pH ≈ 12	3.7(92%)	1.8
TMBDY	DMSO:water (20:80) 79 mM Phe, pH ≈ 12	5.7	
$C_{10}BDYC_{10}$	monoolein:water (70:30 wt%) {cubic phase}	5.0	
$C_{10}BDYC_{10}$	DOPC {vesicles in water}	5.8	
BDYIA	water	6.0	
BDYIA	PAI-1 {in water} active protein	6.5 (95%)	2.9
BDYIA	PAI-1 {in water} latent protein	6.5 (93%)	3.2
BDYIA	PAI-1 {in 50% glycerol} active protein	5.8	

[a] The acronyms for the BODIPY derivatives are 3,3',4,4'-difluoro-1,3,5,7-tetramethyl-4-borato-3a-azonia-4a-aza-*s*-indacene (TMBDY), 3,5-didecyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene ($C_{10}BDYC_{10}$), *N*-(4,4-difluoro-5,7-dimethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-3-propionyl)-*N'*-(iodoacetyl)ethylenediamine (BDYIA). The fluorescence lifetimes were calculated from deconvolutions of data measured by means of the single photon counting technique. The statistical tests (χ square, Durbin–Watson, Null hypothesis) were all accepted for the single or biexponential decays presented.

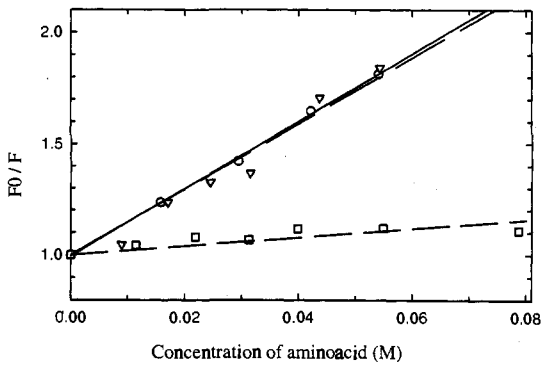

Figure 4. The fluorescence quenching of 3,3',4,4-difluoro-1,3,5,7-tetramethyl-4-borata-3a-azonia-4a-aza-*s*-indacene (TMBDY) in a mixture of water and DMSO (80:20 wt %, pH ≈ 12) by the amino acids Trp (○), Tyr (▽), and Phe (□). The Stern–Volmer plots show the ratio between the fluorescence intensity in the absence (F_0) and presence (F) of the amino acid, as a function of the amino acid concentrations. The Stern–Volmer constants (K) for Trp, Tyr, and Phe are 15, 14, and 1.4 M^{-1}, respectively. The quenching constants, $k_q = K/\langle\tau\rangle$, are 5.4×10^9, 2.5×10^9, and 2.4×10^8 M^{-1} s^{-1} for Trp, Tyr, and Phe, where $\langle\tau\rangle$ denotes the average lifetime.

The limiting *fluorescence anisotropy* (r_0) of BODIPY was determined for TMBDY and BDYSE (4,4-difluoro-5,7-dimethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-1-propionic acid, succimidyl ester) in 1,2-propanediol and glycerol. For both compounds a value of $r_0 = 0.370 \pm 0.002$ was found by lowering the temperature. In glycerol, the r_0 value is reached below 250 K, while TMBDY in 1,2-propanediol reaches this maximum below 220 K. This result is close to that of $r_0 = 0.373 \pm 0.002$, previously obtained for different xanthene derivatives.[17] The experimental r_0 means that the $S_0 \leftrightarrow S_1$ transition dipoles mutually form an effective angle of $\theta \approx 13°$.

The *fluorescence excitation anisotropy* (see Figure 3) is constant (and equal to r_0) across the spectrum of the strong $S_0 \rightarrow S_1$ band, which shows that the transition contains one direction of the electronic transition dipole moment. Across the $S_0 \rightarrow S_2$ band a constant, but slightly lower value of the anisotropy is observed. Since the $S_0 \rightarrow S_2$ transition is much weaker than the $S_0 \rightarrow S_1$ transition the lower value on the anisotropy could be due to vibronic mixing.

BODIPY in Lipid Bilayers. The lipid derivatives $C_{10}BDYC_{10}$ (3,5-didecyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene) and $C_{10}BDYC_3$ (5-decyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indazene-3-propionic acid) were solubilized in lamellar liquid–crystalline phases of pentaethyleneglycol mono-*n*-dodecyl ether ($C_{12}EO_5$), monoolein, and DOPC. The orientation of the BODIPY derivatives in macroscopically aligned lamellar phases was studied by using *LD spectroscopy* and the order parameters (S), corresponding to the $S_0 \rightarrow S_1$ transition, are summarized in Table 2. The positive and low S values obtained for $C_{10}BDYC_3$ show that the long axis of BODIPY tends to be oriented perpendicular to the lipid bilayer. Such a preferential orientation is not surprising, although one might expect higher S values, if the long axis of BODIPY is inserted between the acyl chains in the polar head group region. However, since the BODIPY moiety is slightly soluble in water, the lowered order is likely due to a localization of chromophore toward the water region. This agrees qualitatively with the negative S values obtained for $C_{10}BDYC_{10}$ molecules residing in two different lamellar phases composed of monoolein and DOPC. In DOPC, the long axis of BODIPY orients preferentially in the plane of the lipid bilayer.

Table 2. The Order Parameter (S) Obtained from Linear Dichroism (LD) Experiments on Different Macroscopically Aligned Lamellar Phases[a]

system	probe	S	probe	S
monoolein/water 90:10 wt %	$C_{10}BDYC_{10}$	−0.09	$C_{10}BDYC_3$	0.27
$C_{12}EO_5$/water 69:31 wt %	$C_{10}BDYC_{10}$		$C_{10}BDYC_3$	0.16
DOPC/water 80:20 wt %	$C_{10}BDYC_{10}$	−0.39	$C_{10}BDYC_3$	0.12

[a] The lipid BODIPY probes were 3,5-didecyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene ($C_{10}BDYC_{10}$) and 5-decyl-4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene-3-propionic acid (C_3BDYC_{10}).

The *fluorescence relaxation* of $C_{10}BDYC_{10}$ in a cubic liquid–crystalline phase of monoolein and in lipid vesicles of DOPC were studied. The molar ratios between the probe and the lipid were about $1:10^6$ and 1:5000 for the cubic phase and the lipid vesicles, respectively. Energy migration between the chromophores can be safely neglected at these concentrations. In both systems the fluorescence relaxation can be fitted to a single exponential function (see Table 1). The rotational motions in lipid bilayers occur on the sub- and nanosecond time scales. For example, $r(t) = 0.22 \exp(-t/2.4\text{ ns})$ for $C_{10}BDYC_{10}$ in the cubic phase (composed of monoolein:water = 70:30 wt %, 293 K). This shows that the rotation of $C_{10}BDYC_{10}$ cannot be severly hindered although the chromophore is anchored to the bilayer by two decyl chains. The decay of $r(t)$ and the measured order parameter (see Table 2) strongly indicate a broad orientational distribution of the BODIPY moiety, with respect to the lipid bilayer of monoolein.

From experiments with $C_{10}BDYC_{10}$ in DOPC vesicles at 296 K, we extract an orientational correlation function of $\rho(t) = 0.46 \exp(-t/2.7\text{ ns}) + 0.21$ (±0.03). Here, since $\phi_c \gg \tau$, we can neglect contributions to $r(t)$ from the rotation of the vesicle. Consequently, the BODIPY moiety undergoes rapid unresolved rotational motions and rotations on the nanosecond time scale and finally reaches an equilibrium orientation (*i.e.*, according to eq 7) at times $t > t_\infty \approx 15$ ns. From the value of ρ_∞ we estimate

(17) Johansson, L. B.-A. *J. Chem. Soc., Faraday Trans. 1* **1990**, *86*, 2103.

the order parameter $|S| = 0.43 \pm 0.06$, which is compatible with $S = -0.39$ for $C_{10}BDYC_{10}$ in the lamellar phase of DOPC (see Table 2). The orientation of $C_{10}BDYC_{10}$ is illustrated in Figure 2 where the long axis of BODIPY is preferentially in the plane of the lipid–water interface. One should, however, remember that no conclusion about the preferential orientation of its short axis relative to the normal of the lipid bilayer can be drawn from this S value.

BODIPY in a Protein. The sulfydryl specific reagent *N*-(4,4-difluoro-5,7-dimethyl-4-borata-3a-azonia-4a-aza-*s*-indacene-3-propionyl)-*N'*-(iodoacetyl)ethylenediamine (BDYIA) was covalently linked to the Cys 344 residue in the Ser 344 → Cys mutant of PAI-1. PAI-1 exists either as an active or a latent form.[4] For both forms the absorption (λ_{max} = 506 nm) and fluorescence (λ_{max} = 512 nm) spectra are, except for a small redshift, identical with the spectra recorded for BODIPY in solvents. But, we find that the fluorescence relaxation is much better described by a double exponential than a single exponential function. BDYIA in both active and latent PAI-1 show a dominating lifetime component of $\tau_1 \approx 6.5$ ns and the second one of $\tau_2 \approx 3$ ns. No significant changes of τ_1 and τ_2 (or the preexponential factors) were found in different regions of the fluorescence spectrum. Therefore the biexponentiality is likely not due to a slow environmental response on the excited BODIPY. Further support for this is the single lifetime of $\tau = 5.8$ ns measured as water is partly (50% by volume) replaced by glycerol. It should be noted that PAI-1 activity is *not* affected by adding this amount of glycerol. Another source of explanation is a dynamic quenching by the aromatic amino acids. By examining the X-ray structure of *latent* PAI-1[18] in the region of P3, one finds that the nearest aromatic residue, Phe, is located at about 10 Å from the P3 position (the distance from C_α 362 to C_α Phe 317 is 9.61 Å). It is not possible to exclude a direct contact between this phenyl residue and the BODIPY moiety, but as a source of dynamical quenching, it is not likely for two reasons. Firstly, the photophysics of BODIPY in the active and latent forms remains the same, although a dramatic conformational difference in the P3 region is present.[18,19] Secondly, Phe is not an efficient quencher, as judged from our quenching experiments. At present we are not able to give an unambigous explanation to the biexponential photophysics of BODIPY in PAI-1.

The steady-state and time-resolved fluorescence anisotropies {r_s and $r(t)$} were determined at 277 K. We obtain values on the excitation and emission anisotropies of $r_s(\lambda_{em}) = r_s(\lambda_{em}) = 0.197$. Combining these results with the single photon counting experiments we find the time-resolved fluorescence anisotropy of

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) \qquad (8)$$

where $r_1 = r_0\rho_1 = 0.089 \pm 0.03$, $r_2 = r_0\rho_2 = 0.197 \pm 0.01$, $\phi_1 = 2.1 \pm 0.7$ ns, and $\phi_2 = 47 \pm 12$ ns. The initial value of $r(0) = r_1 + r_2 = 0.286$ is less than its maximum possible value of $r(0) = r_0 = 0.37$, meaning that the fluorophore undergoes fast rotational motions on a time scale beyond the time-resolution of our experiment, *i.e.*, in the order of times < 100 ps. The rotational correlation time $\phi_1 \approx 2$ ns can be ascribed to local mobilities of the BODIPY moiety, that is, $\phi_1 \approx \Phi_1$ in eq 6, while ϕ_2 is explained by an overall rotational tumbling of the protein molecule, that is, $\phi_2 \approx \phi_c$ in eq 4. An estimated rotational correlation time, ϕ_{calc} = 39.4 ns of the active form of PAI-1, can be calculated from the Stokes–Einstein equation with an apparent Stokes' radius of 2.77 nm[19] and the measured solvent viscosity {η(277 K) = 1.69 cP}. Thus, the values of $\phi_2 \approx \phi_c$ extracted from experimental data are compatible with ϕ_{calc}. This suggests that ρ_∞ can be obtained from the value of r_2. However, by increasing the solvent viscosity one expects that $\phi_2 \approx \phi_c \to \infty$, on the time scale of the experiment. This is indeed what is observed upon replacing water with glycerol. We find that $\phi_1 = 6.2 \pm 1.3$ ns, $\phi_2 \gg 50$ ns ($\approx \infty$), $r_1 = 0.117 \pm 0.01$, and $r_2 = 0.202 \pm 0.01 \approx r_0\rho_\infty$. The relatively large value on r_2 implies that the local mobility of the BODIPY moiety is restricted. By assuming a simple wobbling-in-cone model[20] of the BODIPY moiety we can estimate the semicone angle of $\beta_0 = 35°$, from the value on ρ_∞ and eq 7.

Concluding Remarks

The BODIPY molecules are found to be highly fluorescent and insensitive to solvent polarity as well as pH. The strong electronic transition, at about 500 nm, which contains long axis polarized electronic transition dipoles, is safely shifted from overlapping absorption bands from proteins as well as from lipid membranes. Moreover, the Förster radius of 57 Å, as well as, BODIPY's weak dependence on pH and polarity, make BODIPY probes highly suited for investigating the influence of electronic donor–donor transfer over distances, typical for the size of many proteins and the thickness of lipid bilayers. Compared to the family of xanthene dyes (to which rhodamines and fluoresceins belong) the BODIPY chromophore is smaller and more insensitive to environmental conditions, while R_0 has about the same value. Furthermore, the labeling of the Cys mutants of PAI-1, by means of iodoacetamide derivatives of rhodamines was much less efficient than for the corresponding derivatives of BODIPY (unpublished data). Our experiments with mutants of Cys PAI-1, to which BDYIA is covalently linked, show that the biological activity remains unaffected, as compared to wildtype PAI-1. Derivatives of BODIPY linked to proteins will be useful, we believe, in studies such as conformational changes and interactions with other proteins, membranes, *etc.*, and this in particular by utilizing energy migration.

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(18) Mottonen, J.; Strand, A.; Symerskyl, J.; Sweet, R. M.; Daneyl, D. E.; Geoghegan, K. F.; Gerard, R. D.; Goldsmith, E. J. *Nature* **1992**, *355*, 270.

(19) Levin, E. G.; Santell, L. *Blood* **1987**, *70*, 1090.

(20) Kinosita, K., Jr.; Kawato, S.; Ikegami, A. *Biophys. J.* **1977**, *20*, 289.