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Determination of the Orientation Distribution of Adsorbed Fluorophores Using TIRF. I. Theory

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ABSTRACT The spectroscopic technique total internal reflection fluorescence can be used for determination of the orientation of adsorbed fluorescent molecules. The underlying theory is presented in general terms and elaborated in detail for the case that the fluorescent group is a porphyrin ring. It is shown that order parameters of the orientation distribution can be obtained if both the fluorescence intensity and its polarization are measured as functions of the polarization of the incident laser beam. From these order parameters an approximation of the orientation distribution can be derived by the maximum-entropy method.

INTRODUCTION

Insight into the orientation distribution of molecules at solid surfaces is important in many disciplines and for many applications. For example, the optical and electrical properties of polymer and surfactant films strongly depend on the structural organization of the films. The permeability of phospholipid monolayers and bilayers, standing model for biological membranes, is directly related to the degree of order of the layers (Nelson and Leermakers, 1990), and the orientation of light-harvesting molecules (e.g., porphyrins) in solar energy cells determines the efficiency of such devices (Yanagi et al., 1990; Wienke et al., 1994). In medical technology the orientation of adsorbed proteins such as antibodies and enzymes largely determines the sensitivity of immunoassay's and diagnostic tests (Schumann and Schmidt, 1992; Bos, 1994).

A number of techniques are available to study the orientation of adsorbed molecules, among which attenuated total reflection infrared spectroscopy (Song et al., 1992; Mizuta et al., 1993), polarized UV/VIS spectroscopy (Wang et al., 1992; Azumi et al., 1993), x-ray diffraction (Azumi et al., 1993), x-ray photoelectron spectroscopy (Margalit and Vasquez, 1990), and electron paramagnetic resonance (Nordio and Busolin, 1971; Pace et al., 1989; Azumi et al., 1993). Although each of these spectroscopic techniques has its advantages and limitations, the only one that can provide angular-distribution data is electron paramagnetic resonance. The other techniques merely give one order parameter of the orientation distribution of the molecules, or an average orientation angle. An alternative and promising technique is atomic force microscopy. With this technique it is possible to observe directly the topology of the surface with its adsorbed molecules (Schwartz et al., 1992; Kajiyama et al., 1994).

Total internal reflection fluorescence (TIRF) can provide information on the orientation of fluorescent or fluorescent-labeled molecules at optically transparent solid surfaces. By variation of the polarization angle Ψ of the incident light beam, the direction of the electric field component of the evanescent field is modified. As a result the interaction between the transition dipole moments of the molecules and the evanescent wave alters, which, in turn, gives rise to a change in the fluorescence signal. The method was first used by Thompson et al. (1984) to detect the order in supported layers of fluorescent-labeled phospholipids. Fraaije et al. (1990) conducted TIRF measurements on adsorbed cytochrome *c* molecules, using the haem group as an intrinsic fluorescent label.

Inasmuch as fluorescence is a two-photon process, it is inherent in the TIRF method that more than one order parameter of the orientation distribution can be obtained (Thompson and Burghardt, 1986). However, if only the intensity of the fluorescence as a function of Ψ is measured, as was done by Thompson et al. (1984) and Fraaije et al. (1990), the information obtained remains limited to an average orientation angle.

We show that, by measuring not only the intensity but also the polarization of the fluorescence as a function of the polarization of the incident light beam, all the principal available information on the orientation distribution can be retrieved. In the first section the theory is presented in general terms, and subsequently it is elaborated in detail for the case the fluorescent group is a porphyrin. By using the maximum-entropy method, orientation distribution functions can be reconstructed. In the last section the influence of the rotational mobility of the adsorbed molecules on the orientation measurements is briefly evaluated. In an accompanying paper (Bos and Kleijn, 1995) the applicability of the presented theory is demonstrated by measurements on adsorbed porphyrin and cytochrome *c* molecules.

GENERAL THEORETICAL BASIS

A light beam striking a planar interface between two media 1 and 2 with refractive indices n_1 and n_2 is totally reflected if $n_1 > n_2$ and if the angle of incidence θ_i exceeds the critical

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angle θ_c, defined by $\theta_c = \arcsin(n_2/n_1)$. Because of interference of the incident and reflected light beams a standing wave is generated in the optically denser medium. As a result an electromagnetic field exists in the rarer medium beyond the reflecting interface, called the evanescent field. Its amplitude decays exponentially with distance from the interface.

In TIRF experiments usually the denser medium is glass or quartz and the rarer medium is a solution or gas phase. The penetration depth of the evanescent field, i.e., the distance over which its amplitude decreases by a factor $1/e$, is typically of the order of 100 nm. Therefore, only fluorescent molecules at or near the interface are excited. Assuming that the fluorescence lifetime of the molecules is much shorter than their rotational correlation time and that energy transfer between the molecules is negligible, the total detected fluorescence is given by

$$F = C\langle(\boldsymbol{\mu}\cdot\mathbf{E})^2 f(\boldsymbol{\nu})\rangle, \tag{1}$$

where $\boldsymbol{\mu}$ and $\boldsymbol{\nu}$ represent the directions of the absorption and emission transition dipoles moment of the molecule, respectively, **E** stands for the direction of the electric field component of the evanescent field, and $f(\boldsymbol{\nu})$ is the collection efficiency of the emitted light. The constant C incorporates the magnitudes of the absorption and emission dipole moments, the quantum yield, the surface concentration of fluorophores, the intensity of the evanescent field, and properties of the detection system. The directions of the transition dipole moments $\boldsymbol{\mu}$ and $\boldsymbol{\nu}$ depend of course on the orientation Ω of the fluorescent group. The angle brackets $\langle\,\rangle$ in Eq. 1 denote an average over all abundant orientations of the fluorescent groups in the sample:

$$\langle x\rangle \equiv \int_{\Omega} xN(\Omega)\,\mathrm{d}\Omega, \tag{2}$$

with $N(\Omega)$ their normalized distribution function.

The direction of the electric field vector of the evanescent field, **E**, can be varied in a simple way by changing the polarization angle Ψ of the incident light beam (Harrick, 1967). Therefore, by using different polarizations of the incident light, it is possible to obtain information on the orientation of the molecules near the interface. Defining an orthogonal coordinate system in which the xy plane corresponds to the interface, the direction of **E** can be given in terms of the relative magnitudes of its x, y, and z components. These vary with the polarization of the incident light beam in the following way (Harrick, 1967):

$$E_x = \epsilon_x \cos\Psi, \tag{3a}$$

$$E_y = \epsilon_y \sin\Psi, \tag{3b}$$

$$E_z = \epsilon_z \cos\Psi. \tag{3c}$$

For total internal reflection at the interface between two media, ϵ_x, ϵ_y, and ϵ_z depend on the refractive indices of these media and on the angle of incidence as described by Harrick (1967); for a multilayer system they depend also on the thicknesses of the different layers and can be calculated by using Hansen's method based on the matrix formalism of Abeles (Hansen, 1968).

The detected fluorescence per molecule is a function of the direction of the emission dipole moment ν, the direction of detection and the aperture angle of the detection system. For a pulsed excitation of the fluorophores and detection along the normal of the interface, here defined as the z axis, the collection efficiency is given by (Axelrod, 1979; Burghardt and Thompson, 1984)

$$f(\boldsymbol{\nu}) = (\nu_x^2 + \nu_y^2) + (1-\gamma)\nu_z^2. \tag{4}$$

The parameter γ is the so-called dichroic factor. The relation between γ and the aperture angle of the detection system is given by Burghardt and Thompson (1984). Equation 4 has been derived by treating the emission dipole as a fixed-amplitude dipole oscillator. For continuously illuminated fluorophores a fixed-power dipole model is more appropriate (Hellen and Axelrod, 1987).

Substitution of Eqs. 3 and 4 into Eq. 1 and using appropriate expressions for the transition dipole moments $\boldsymbol{\mu}$ and $\boldsymbol{\nu}$ lead to an equation of the general form

$$F(\Psi) = C(A + B\cos^2\Psi), \tag{5}$$

in which A and B are functions of ϵ_x, ϵ_y, and ϵ_z, the dichroic factor γ, and order parameters of the orientation distribution of the fluorophores. Generally, the value of the proportionality constant C will be unknown, because it depends on many variables, some of them varying with the experiment and difficult to assess. Therefore, by measuring the fluorescence intensity as a function of the polarization angle of the incident light, $F(\Psi)$, only the ratio A/B will be found, from which an average orientation angle can be derived. By measuring not only the intensity but also the polarization of the fluorescence it is possible to determine all the order parameters for which the fluorescence signal is sensitive. From these order parameters an approximation of the orientation distribution $N(\Omega)$ can be obtained. This will be elaborated for the case of orientation measurements on porphyrins.

ORIENTATION MEASUREMENTS ON PORPHYRINS

In Fig. 1 the structure of the free-base porphyrin ring is shown. Unlike for the metal porphyrins, there are distinct X- and Y-symmetry axes. By definition the molecular X axis passes through the two central protons (Gouterman and Stryer, 1962). This structure has a characteristic four-banded visible absorption spectrum (see Fig. 2 (*a*)). The Q bands represent absorption along two transition dipole moments oriented in the plane of the ring along the molecular X and Y axes, $\boldsymbol{\mu}_X$ and $\boldsymbol{\mu}_Y$ (Gouterman and Stryer, 1962; Vanderkooi and Erecinska, 1975; Vanderkooi et al., 1976). Emission takes place along two transition dipole moments $\boldsymbol{\nu}_X$ and $\boldsymbol{\nu}_Y$ parallel to the absorption dipole moments. The emission spectrum is shown in Fig. 2 (*b*).

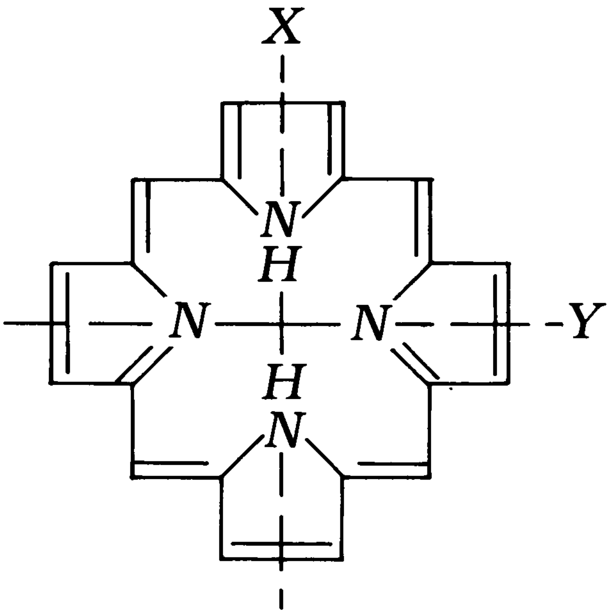

FIGURE 1 Structure of the free-base porphyrin ring, in which the molecular X and Y axes are indicated.

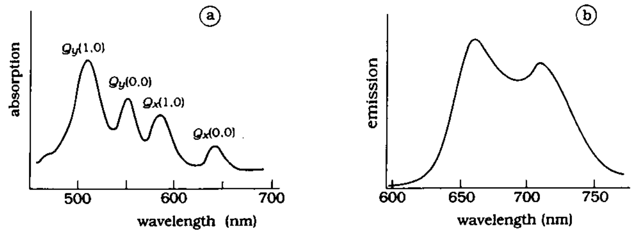

FIGURE 2 Typical absorption and emission spectra of a porphyrin.

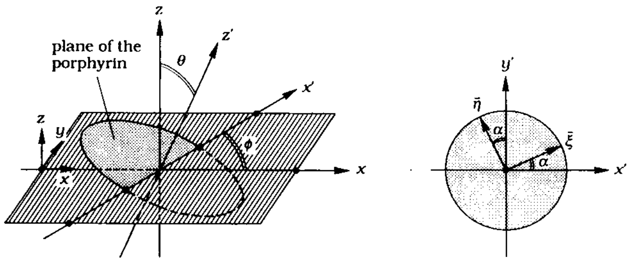

FIGURE 3 Definition of the coordinate systems used in the theory. The interface is (parallel to) the xy-plane. The $x'y'$ plane corresponds to the plane of the porphyrin. The directions of the transition dipole moments in the porphyrin plane are defined by the three orientation angles α, ϕ, and θ.

We now introduce a second orthogonal coordinate system $x'y'z'$ to describe the absorption and emission transition dipole moments in the porphyrin ring system. In this coordinate system the $x'y'$ plane corresponds to the plane of the porphyrin ring. In Fig. 3 the relation between the new coordinate system $x'y'z'$ and the laboratory coordinate system xyz is shown; the x' axis is taken parallel to the interface, i.e., in the xy plane. A complication is that the central protons in the porphyrin ring can move over to the other pair of opposite pyrrole rings, resulting in a continuous exchange of the orientations of the molecular X and Y axes. This tautomeric process (time scale microseconds (Janson and Katz, 1979)) is much slower than the absorption and emission process (time scale nanoseconds (Gouterman and Stryer, 1962; Vanderkooi and Erecinska, 1975)). As a consequence the X and Y axes and the transition dipole moments $\boldsymbol{\mu}_X$, $\boldsymbol{\mu}_Y$, $\boldsymbol{\nu}_X$, and $\boldsymbol{\nu}_Y$ do not have fixed orientations in the molecule. However, we can define two auxiliary vectors $\boldsymbol{\xi}$ and $\boldsymbol{\eta}$ that do have fixed orientations in the molecule, representing the directions of the molecular axes. The directions of $\boldsymbol{\xi}$ and $\boldsymbol{\eta}$ in the laboratory coordinate system xyz are defined by three orientation angles θ, ϕ, and α: θ is the angle between the z' axis and the z axis (i.e., the tilt angle between the porphyrin ring and the interface); ϕ is the angle between the x' axis and the x axis; and α is the angle between $\boldsymbol{\xi}$ and the x' axis, which is equal to the angle between $\boldsymbol{\eta}$ and the y' axis. In this way the following expressions for $\boldsymbol{\xi}$ and $\boldsymbol{\eta}$ in the laboratory coordinate system are found:

$$\boldsymbol{\xi} = \begin{pmatrix} \cos\phi\cos\alpha - \cos\theta\sin\phi\sin\alpha \\ \sin\phi\cos\alpha + \cos\theta\cos\phi\sin\alpha \\ \sin\theta\sin\alpha \end{pmatrix}, \tag{6a}$$

$$\boldsymbol{\eta} = \begin{pmatrix} -\cos\phi\sin\alpha - \cos\theta\sin\phi\cos\alpha \\ -\sin\phi\sin\alpha + \cos\theta\cos\phi\cos\alpha \\ \sin\theta\cos\alpha \end{pmatrix}. \tag{6b}$$

The orientation distribution of the porphyrin rings at the interface is given by a function $N(\theta, \phi, \alpha)$, which is normalized so that

$$\int_{\theta=0}^{\pi}\int_{\phi=0}^{2\pi}\int_{\alpha=0}^{2\pi} N(\theta, \phi, \alpha)\sin\theta\, d\alpha\, d\phi\, d\theta = 1. \tag{7}$$

The absorption and emission bands shown in Fig. 2 are of a composite nature (Gouterman and Stryer, 1962); i.e., depending on the wavelength and the bandwidth of the excitation light, absorption takes place partly along $\boldsymbol{\mu}_X$ (this fraction is called r_X) and partly along $\boldsymbol{\mu}_Y$ (fraction r_Y), and, depending on the detection wavelength and bandwidth, the detected emission stems partly from $\boldsymbol{\nu}_X$ (fraction q_X) and partly from $\boldsymbol{\nu}_Y$ (fraction q_Y). Thus, the measured fluorescence signal consists of four contributions, $F(\boldsymbol{\mu}_X, \boldsymbol{\nu}_X)$, $F(\boldsymbol{\mu}_X, \boldsymbol{\nu}_Y)$, $F(\boldsymbol{\mu}_Y, \boldsymbol{\nu}_X)$, and $F(\boldsymbol{\mu}_Y, \boldsymbol{\nu}_Y)$, with relative intensities r_Xq_X, r_Xq_Y, r_Yq_X, and r_Yq_Y, respectively:

$$F = r_Xq_XF(\boldsymbol{\mu}_X, \boldsymbol{\nu}_X) + r_Xq_YF(\boldsymbol{\mu}_X, \boldsymbol{\nu}_Y) + r_Yq_XF(\boldsymbol{\mu}_Y, \boldsymbol{\nu}_X) + r_Yq_YF(\boldsymbol{\mu}_Y, \boldsymbol{\nu}_Y), \tag{8}$$

in which, in accordance with Eq. 1,

$$F(\boldsymbol{\mu}_X, \boldsymbol{\nu}_X) = C\langle(\boldsymbol{\mu}_X \cdot \mathbf{E})^2 f(\boldsymbol{\nu}_X)\rangle, \text{ etc.}$$

By taking for all four contributions to F the same proportionality constant C, we implicitly assume that the magnitudes of the absorption dipole moments along the molecular X axis and Y axis are equal and also that the magnitudes of the emission dipole moments $\boldsymbol{\nu}_X$ and $\boldsymbol{\nu}_Y$ are the same. Because of the distinct *X-Y* symmetry in the porphyrin ring this is not entirely true. Furthermore, an asymmetric environment of the porphyrin ring, as in cytochrome *c*, in which the porphyrin ring is situated at one side of the protein molecule, might result in larger differences in the magnitudes of the transition dipole moments. (In fact, then the magnitude of each individual transition dipole moment will also depend on the tautomeric form in which the porphyrin exists at a particular moment.) However, from the composite character of the absorption bands as well as the emission bands in the spectra of free-base porphyrins and free-base cytochrome *c* (Gouterman and Stryer, 1962; Vanderkooi and Erecinska, 1975) it can be concluded that the differences in magnitude between $\boldsymbol{\mu}_X$ and $\boldsymbol{\mu}_Y$, and also between $\boldsymbol{\nu}_X$ and $\boldsymbol{\nu}_Y$, are small: otherwise mixing would not occur. Making use of the reasonable assumption that for half of the ensemble $\boldsymbol{\mu}_X = \boldsymbol{\nu}_X = \boldsymbol{\xi}$ and $\boldsymbol{\mu}_Y = \boldsymbol{\nu}_Y = \boldsymbol{\eta}$, and for the other half of the molecules $\boldsymbol{\mu}_X = \boldsymbol{\nu}_X = \boldsymbol{\eta}$ and $\boldsymbol{\mu}_Y = \boldsymbol{\nu}_Y = \boldsymbol{\xi}$, we can write

$$F = \frac{1}{2}\{(r_X q_X + r_Y q_Y)[F(\boldsymbol{\xi}, \boldsymbol{\xi}) + F(\boldsymbol{\eta}, \boldsymbol{\eta})] + (r_X q_Y + r_Y q_X)[F(\boldsymbol{\eta}, \boldsymbol{\xi}) + F(\boldsymbol{\xi}, \boldsymbol{\eta})]\}. \tag{9}$$

Equation 9 can be elaborated into a rather complicated expression with many terms. It can be simplified by assuming that the orientation distribution of the molecules at the interface is isotropic in ϕ. This assumption holds if the interface is isotropic in the *x* and *y* directions and if the correlation length of local ordering of the molecules along the angle ϕ is much smaller than the dimensions of the illuminated spot (the area of the interface under examination). The orientation distribution function of the molecules $N(\theta, \phi, \alpha)$ is now replaced by a normalized distribution function $N(\theta, \alpha)$ (for an isotropic distribution in ϕ: $N(\theta, \phi, \alpha) = N(\theta, \alpha)/2\pi$). Using

$$r_X q_X + r_Y q_Y = a, \tag{10a}$$

$$r_X q_Y + r_Y q_X = b, \tag{10b}$$

$$a + b = 1, \tag{10c}$$

we arrive at the following expression for F:

$$F = C[E_x^2 + E_y^2 - \frac{1}{2}\{(\gamma + 1)(E_x^2 + E_y^2) - 2E_z^2\}\langle\sin^2\theta\rangle + \frac{1}{2}a\gamma(E_x^2 + E_y^2 - 2E_z^2)\langle\sin^4\theta\rangle + \gamma(b - a)(E_x^2 + E_y^2 - 2E_z^2)\langle\sin^4\theta\cos^2\alpha\sin^2\alpha\rangle], \tag{11}$$

in which

$$\langle x\rangle = \int_{\theta=0}^{\pi}\int_{\alpha=0}^{2\pi} x\, N(\theta, \alpha)\sin\theta \mathrm{d}\alpha\, \mathrm{d}\theta. \tag{12}$$

Equation 11 can be rewritten, using Eqs. 3, in the general form of Eq. 5. It is obvious that measurement of $F(\Psi)$ does not lead to insight into the orientation distribution of the molecules. However, by measuring the parallel and perpendicular polarized components of the fluorescence, $F_\|(\Psi)$ and $F_\perp(\Psi)$, it is possible to determine $\langle\sin^2\theta\rangle$, $\langle\sin^4\theta\rangle$, and $\langle\sin^4\theta\cos^2\alpha\sin^2\alpha\rangle$. Making use of the following expressions for the collection efficiencies $f_\|$ and $f_\perp$ (Axelrod, 1979):

$$f_\| = \nu_x^2 + \frac{1}{2}(1 - \gamma)\nu_z^2, \tag{13a}$$

$$f_\perp = \nu_y^2 + \frac{1}{2}(1 - \gamma)\nu_z^2 \tag{13b}$$

in the same way as for $F(\Psi)$, we find the following expressions for $F_\|(\Psi)$ and $F_\perp(\Psi)$:

$$F_\| = C\Big[\frac{1}{4}\{(2a + 1)E_x^2 + (2b + 1)E_y^2\} - \frac{1}{4}\{(\gamma + 2a)E_x^2 + (\gamma + 2b)E_y^2 - 2E_z^2\}\langle\sin^2\theta\rangle + \frac{1}{8}a\{(2\gamma + 1)E_x^2 + (2\gamma - 1)E_y^2 - 4\gamma E_z^2\}\langle\sin^4\theta\rangle + \frac{1}{4}(b - \mathrm{a})\{(2\gamma + 1)E_x^2 + (2\gamma - 1)E_y^2 - 4\gamma E_z^2\}\langle\sin^4\theta\cos^2\alpha\sin^2\alpha\rangle\Big], \tag{14a}$$

$$F_\perp = C\Big[\frac{1}{4}\{(2b + 1)E_x^2 + (2a + 1)E_y^2\} - \frac{1}{4}\{(\gamma + 2b)E_x^2 + (\gamma + 2a)E_y^2 - 2E_z^2\}\langle\sin^2\theta\rangle + \frac{1}{8}a\{(2\gamma - 1)E_x^2 + (2\gamma + 1)E_y^2 - 4\gamma E_z^2\}\langle\sin^4\theta\rangle + \frac{1}{4}(b - a)\{(2\gamma - 1)E_x^2 + (2\gamma + 1)E_y^2 - 4\gamma E_z^2\}\langle\sin^4\theta\cos^2\alpha\sin^2\alpha\rangle\Big]. \tag{14b}$$

Like $F(\Psi)$ these polarized components of the fluorescence are linear functions of $\cos^2\Psi$:

$$F_\|(\Psi) = C(A_\| + B_\|\cos^2\Psi), \tag{15a}$$

$$F_\perp(\Psi) = C(A_\perp + B_\perp\cos^2\Psi). \tag{15b}$$

Now three independent parameters (viz., the ratios of $A_\|$, $A_\perp$, $B_\|$, and $B_\perp$) can be determined, from which the values of $\langle\sin^2\theta\rangle$, $\langle\sin^4\theta\rangle$, and $\langle\sin^4\theta\cos^2\alpha\sin^2\alpha\rangle$ can be calculated. Because $F_\|(\Psi)$ and $F_\perp(\Psi)$ are linear functions of $\cos^2\Psi$, four measurements, for example $F_\|(0°)$, $F_\perp(0°)$, $F_\|(90°)$, and $F_\perp$ (90°), are sufficient to extract the available information on the orientation distribution of the molecules.

The values for the parameters a and b depend on the excitation and detection wavelengths and bandwidths applied and can be obtained from polarization spectra. It can be shown (Gouterman and Stryer, 1962) that for porphyrins with distinct X-Y symmetry axes the relation between the polarization P and the fractions of absorption and emission along these two axes is given by

$$\frac{3P+1}{3-P} = r_X q_X + r_Y q_Y. \tag{16}$$

For tetraphenylporphyrin and for porphyrin cytochrome c (the free-base derivate of cytochrome c) in highly viscous media, polarization spectra have been obtained by Gouterman and Stryer (1962) and Vanderkooi and Erecinska (1975), respectively. From the data provided by these authors it can be derived that for both molecules in the $Q_y(1, 0)$ transition band (see Fig. 2) 50% of the absorption takes place along the X axis and 50% along the Y axis, i.e., $r_X = 0.5$ and $r_Y = 0.5$. This implies that $a = b = 0.5$, independent of the wavelength and bandwidth of detection. Thus, for excitation in the $Q_y(1, 0)$ band the expressions for $F_{\|}(0°)$, $F_{\perp}(0°)$, $F_{\|}(90°)$, and $F_{\perp}(90°)$ become

$$\begin{aligned} F_{\|}(0°) = \frac{1}{4}C\Big[&2\epsilon_x^2 - \{(\gamma+1)\epsilon_x^2 - 2\epsilon_z^2\}\langle\sin^2\theta\rangle \\ &+ \frac{1}{4}\{(2\gamma+1)\epsilon_x^2 - 4\gamma\epsilon_z^2\}\langle\sin^4\theta\rangle\Big], \end{aligned} \tag{17a}$$

$$\begin{aligned} F_{\|}(90°) = \frac{1}{4}C\Big[&2\epsilon_y^2 - (\gamma+1)\epsilon_y^2\langle\sin^2\theta\rangle \\ &+ \frac{1}{4}(2\gamma-1)\epsilon_y^2\langle\sin^4\theta\rangle\Big], \end{aligned} \tag{17b}$$

$$\begin{aligned} F_{\perp}(0°) = \frac{1}{4}C\Big[&2\epsilon_x^2 - \{(\gamma+1)\epsilon_x^2 - 2\epsilon_z^2\}\langle\sin^2\theta\rangle \\ &+ \frac{1}{4}\{(2\gamma-1)\epsilon_x^2 - 4\gamma\epsilon_z^2\}\langle\sin^4\theta\rangle\Big], \end{aligned} \tag{17c}$$

$$\begin{aligned} F_{\perp}(90°) = \frac{1}{4}C\Big[&2\epsilon_y^2 - (\gamma+1)\epsilon_y^2\langle\sin^2\theta\rangle \\ &+ \frac{1}{4}(2\gamma+1)\epsilon_y^2\langle\sin^4\theta\rangle\Big]. \end{aligned} \tag{17d}$$

It is a direct consequence of the equality $a = b = 0.5$ that the orientation angle α no longer occurs in the expressions for the fluorescence. Therefore, by using an excitation wavelength in the Q_y (1, 0) band, only information on the orientation in θ is obtained, i.e., the tilt angle of the porphyrin plane.

ORIENTATION DISTRIBUTION FUNCTION

The orientation distribution function $N(\phi, \alpha)$ of the porphyrin groups can be expanded in a set of orthogonal functions, the spherical harmonics $Y_{l,m}(\theta, \alpha)$ (Atkins, 1983):

$$N(\theta, \alpha) = \sum_l \sum_m d_{l,m} Y_{l,m}(\theta, \alpha). \tag{18}$$

The coefficients $d_{l,m}$ are given by

$$d_{l,m} = \int_0^{\pi}\int_0^{2\pi} N(\theta, \alpha) Y_{l,m}^*(\theta, \alpha) \sin\theta \, d\alpha \, d\theta, \tag{19}$$

where $Y_{l,m}^*$ is the complex conjugate of $Y_{l,m}$. The distribution function is fully characterized if all the coefficients $d_{l,m}$ are known. However, from experiments only the values of $\langle\sin^2\theta\rangle$, $\langle\sin^4\theta\rangle$, and $\langle\sin^4\theta\cos^2\alpha\sin^2\alpha\rangle$ can be obtained, and these are directly related to $d_{2,0}$, $d_{4,0}$, and $d_{4,\pm 4}$; the other coefficients of the expansion do not affect $F(\Psi)$ or its polarized components.

In the case of excitation in the $Q_y(1, 0)$ band, only information on the orientation distribution in θ is obtained. Now, the orientation distribution $N(\theta)$ can be expanded in Legendre polynomials:

$$N(\theta) = \sum_{n=0}^{\infty} c_n P_n(\cos\theta), \tag{20}$$

in which

$$c_n = \left[\frac{(2n+1)}{2}\right]\langle P_n\rangle. \tag{21}$$

$\langle P_n\rangle$ are the momenta of the distribution function, the so-called order parameters. The orientation distribution in θ is fully characterized if all the order parameters $\langle P_n\rangle$ are known. As a result of the symmetry in the porphyrin ring there is no difference between $N(\theta)$ and $N(\pi - \theta)$, corresponding to the uneven coefficients c_n being zero. Experimentally only two order parameters are accessible, i.e., $\langle P_2\rangle$ and $\langle P_4\rangle$. $\langle P_2\rangle$ and $\langle P_4\rangle$ are given by

$$\langle P_2\rangle = \frac{1}{2}(3\langle\cos^2\theta\rangle - 1), \tag{22a}$$

$$\langle P_4\rangle = \frac{1}{8}(35\langle\cos^4\theta\rangle - 30\langle\cos^2\theta\rangle + 3), \tag{22b}$$

which can be calculated from $\langle\sin^2\theta\rangle$ and $\langle\sin^4\theta\rangle$. From Eq. 22a it follows that $\langle P_2\rangle$ should lie between −0.5 and 1.0. Because $\langle\cos^4\theta\rangle \leq \langle\cos^2\theta\rangle$ and $\langle\cos^4\theta\rangle \geq \langle\cos^2\theta\rangle^2$, for a given value of $\langle P_2\rangle$ the value of $\langle P_4\rangle$ should lie between an upper and a lower boundary. This is illustrated in Fig. 4. The upper boundary is the line where $\langle\cos^4\theta\rangle = \langle\cos^2\theta\rangle$; at the lower boundary $\langle\cos^4\theta\rangle = \langle\cos^2\theta\rangle^2$.

One way to reconstruct $N(\theta)$ from the restricted knowledge of the order parameters is to use a model distribution. Because such a model makes a definite relation between $\langle P_2\rangle$ and $\langle P_4\rangle$, its validity follows to some extent from the experimental results. For example, in Fig. 4 the relation between $\langle P_2\rangle$ and $\langle P_4\rangle$ for a Gaussian distribution is given by the curve indicated by $\lambda_4 = 0$. Nevertheless, even if the combination of order parameters does comply with the model used, this method still involves a subjective judgment of the shape of the distribution.

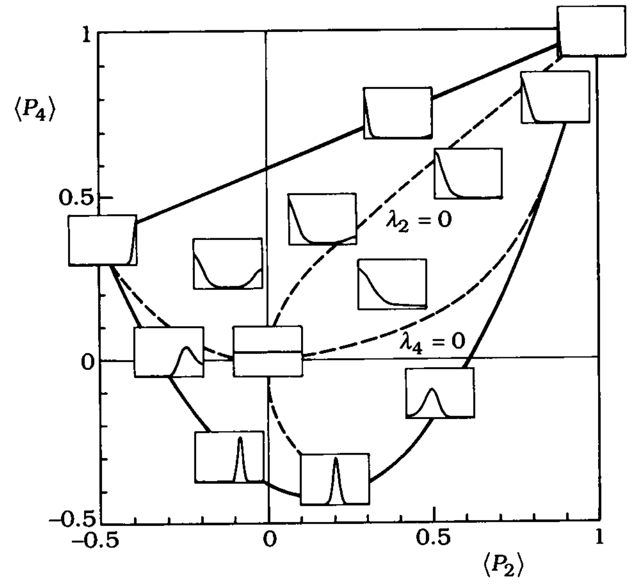

FIGURE 4 Interrelation between the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$. The physical boundaries of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are indicated by solid curves. The insets show the shape of the distribution function $N(\theta)$ for θ between 0° and 90° as calculated following the maximum-entropy method.

Another way to obtain an approximation of $N(\theta)$ is to use the maximum-entropy method, which stems from information theory (Bevensee, 1983). In this approach no a priori assumptions are made concerning the shape of the distribution function (except for it being continuous). The underlying idea is that the most probable distribution function is the one that can be realized in the greatest number of distinct ways, subject to the known constraints. Following the maximum-entropy method, the smoothest and broadest distribution consistent with the limited data at our disposal, and maximally noncommittal with regard to unavailable data, is obtained. The equation for the total number of microstates W that give a particular distribution $N(\theta)$ is

$$W = \frac{n!}{n_1!n_2!\ldots n_i!\ldots} = \frac{n!}{\prod n_i!}, \qquad n = \sum n_i, \tag{23}$$

with n_i the number of molecules with orientation angle θ_i and n the total number of molecules. The most probable distribution function is found by maximizing W, or ln W, which gives the same result but is considerably simpler because then the Stirling approximation for $n \gg 1$ can be used:

$$\ln W = \ln(n!) - \sum_i \ln(n_i!) \approx n \ln n - \sum_i n_i \ln n_i = -\sum_i n_i \ln \frac{n_i}{n}. \tag{24}$$

Because the distribution is a continuous function, the summation over i can be replaced by an integration over θ with the appropriate weight function (sin θ); n_i/n corresponds to $N(\theta)$. We define (ln W)/n as the entropy S of the normalized distribution function:

$$S\{N(\theta)\} = -\int_0^{\pi} N(\theta)\ln\{N(\theta)\} \sin\theta \, d\theta. \tag{25}$$

The maximum of this function, taking into account the constraints of our knowledge, can be found by using the method of Lagrange multipliers (Bevensee, 1983; Van Langen et al., 1989). The result is a Maxwell–Boltzmann distribution with ordering energy $U(\theta)$:

$$N(\theta) = \exp(-U(\theta)/kT) = N_0\exp\{\lambda_2 P_2(\cos\theta) + \lambda_4 P_4(\cos\theta)\}, \tag{26}$$

with N_0 the normalization factor. By fitting λ_2 and λ_4, using a least-squares method, to the experimentally accessible values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ a unique fit is obtained, i.e., each pair $\{\lambda_2, \lambda_4\}$ defines a unique pair of order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$.

In Fig. 4 the shape of the distribution function $N(\theta)$ obtained with the maximum-entropy method as a function of the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ is shown. For $\lambda_4 > 0$ bimodal distributions are found (having maxima at $\theta = 0$ and $\theta = \pi/2$ and a minimum at some intermediate angle), and for $\lambda_4 \leq 0$ we find distributions with one peak. On the line $\langle \cos^4 \theta \rangle = \langle \cos^2 \theta \rangle^2$ relatively sharp distributions around an angle $\theta = \arccos(\langle \cos^2 \theta \rangle^{1/2})$ are found. If both order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are zero, we are dealing with a random distribution.

CONCLUDING REMARKS

The TIRF method for determination of the orientation distribution can be applied to any kind of fluorescent or fluorescent-labeled molecule at an optically transparent flat surface, provided that the basic assumptions made in this paper hold. For molecules with one absorption and one emission dipole moment the angle between these transition dipole moments can also be deduced from the TIRF orientation measurements. This might be useful, for example, when one is using diphenyl hexatriene probes to establish the order in phospholipid layers; the angle between the absorption and emission dipoles in diphenyl hexatriene is dependent on environmental conditions (Van Langen et al., 1987).

One of the basic assumptions in the theory is that the orientation of the adsorbed molecules does not change on the time scale of fluorescence. To assess the effect of rotational mobility on the orientation measurements a model to describe the rotational motion of the molecules is required, e.g., small step stochastic diffusion in an orienting potential (Nordio and Busolin, 1971). It can be shown that, in the limiting case that the rotational mobility of the molecules is much faster than the fluorescence lifetime, polarization of the fluorescence is completely lost, and only the order parameter $\langle P_2 \rangle$ can be retrieved. A way to establish experimentally whether the rotational correlation time of the molecules is in the same order as their fluorescence lifetime is to vary the viscosity of the solution phase. Then the difference between the parallel and perpendicular polarized components of the fluorescence will vary with the viscosity.

Of course, the orientation distribution of the fluorescent groups reflects the orientation distribution of the complete molecules only if the fluorescent group is firmly anchored in the molecule and if the molecule does not change its conformation upon adsorption. Therefore, it is better not to use extrinsic fluorescent labels, unless it is certain that the label is fixed in the molecule with a known orientation. One should always be aware that introducing a fluorescent label might influence the interfacial behavior (including the orientation) of the molecules. A general problem in determining the orientation of adsorbed protein molecules is that most proteins change their conformation upon adsorption, especially on hydrophobic surfaces (Norde, 1992).

In an accompanying paper (Bos and Kleijn, 1995) TIRF orientation measurements on adsorbed tetramethylpyridinium porphyrin and cytochrome *c* molecules are described and the applicability of the theory presented here is evaluated.

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