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The orientation of transition moments of dye molecules used in fluorescence studies of muscle systems

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Abstract. Fluorescence and phosphorescence depolarization techniques can provide information on orientational order and rotational motion of crossbridges in muscle fibres. However the depolarization experiment monitors the orientation and motion of the crossbridges indirectly. The changes in depolarization arise from a change in the orientation of the transition dipoles of the dye attached to the crossbridge. In order to extract the physiologically relevant orientations from the data it is therefore necessary to characterize the orientation of the dye molecule relative to the crossbridge and the orientation of the transition moments in the frame of the dyes. The dyes 1,5-I-AEDANS and eosin-5-maleimide are commonly used for labelling the crossbridge in muscle fibres. The orientations of the absorption and fluorescence emission dipoles of these two dyes in the molecular frame were determined. Angle resolved fluorescence depolarization experiments on the dyes, macroscopically aligned in a stretched polymer matrix of poly vinyl alcohol, were carried out. The data were analyzed in terms of an orientational distribution of the dye molecules in the film and the orientations of the absorption and emission dipoles in the frame of the dye molecule. Experimental data, obtained from a given sample at different excitation wavelengths, were analyzed simultaneously in a global target approach. This leads to a reduction in the number of independent parameters optimized by the non-linear least squares procedure.

Key words: Muscle – Myosin – Fluorescence – Transition dipoles

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Abbreviations: 1,5-I-AEDANS: 5-iodoacetamido-ethyl-amino-naphthalene-a-sulfonic acid; IATR: iodoacetamido-tetra-methyl-rhodamine; E5M: Eosin-5-Maleimide; ATP: adenosine tri phosphate; ε-ATP: 1:N^6-ethano-ATP; ε-2-aza-ATP: 1:N^6-etheno-2-aza-ATP; ant-ATP: anthraniloyl-ATP

Introduction

The contractile force in muscle is generally believed to arise from the cyclic interaction of myosin crossbridges with F-actin, involving the hydrolysis of ATP (A. F. Huxley 1957, H. E. Huxley 1957). In order to gain insight into the molecular mechanism of muscle contraction, much attention has been paid to the orientation of the crossbridges relative to the actin filament and the change of this orientation at different stages in the cycle. A variety of techniques, such as X-ray diffraction, NMR, ESR and optical spectroscopy have been utilized to this end (Squire 1990).

The orientational order and rotational dynamics of dyes incorporated in biological systems such as lipid membranes (Deinum et al. 1988) and muscle fibres (Cooke 1986; Thomas 1987; Ludescher 1990) are conveniently studied using optical techniques. However, these studies require the presence of dye molecules at specific sites in the system. A variety of dye molecules have been used in studies of muscle fibres using optical techniques. While intrinsic fluorophores such as tryptophan (Aronson and Morales 1969) have been used in the past, current studies widely use extrinsic dyes. 1,5-I-AEDANS [1], and IATR are examples of fluorescent molecules that covalently bind to thiol-groups, particularly the SH_1 on the myosin subfragment S_1 (Borejdo and Putnam 1977; Wilson and Mendelson 1983; Ajtaj and Burghardt 1989). The dye E5M also binds to SH_1 and is important for time resolved phosphorescence experiments on the microsecond timescale (Kinosita et al. 1984; Ludescher and Thomas 1988). Furthermore, fluorescent ATP-analogues such as ε-ATP, ε-2-aza-ATP and ant-ATP have been used to probe the ATP-binding site on S_1 (Yanagida 1981, 1985).

The molecular behaviour of the crossbridges can only be extracted from fluorescence or phosphorescence depolarization experiments on muscle fibres using a model describing their orientational order and dynamics. A fluorescence or phosphorescence depolarization experiment however, monitors the behaviour of the crossbridges indi-

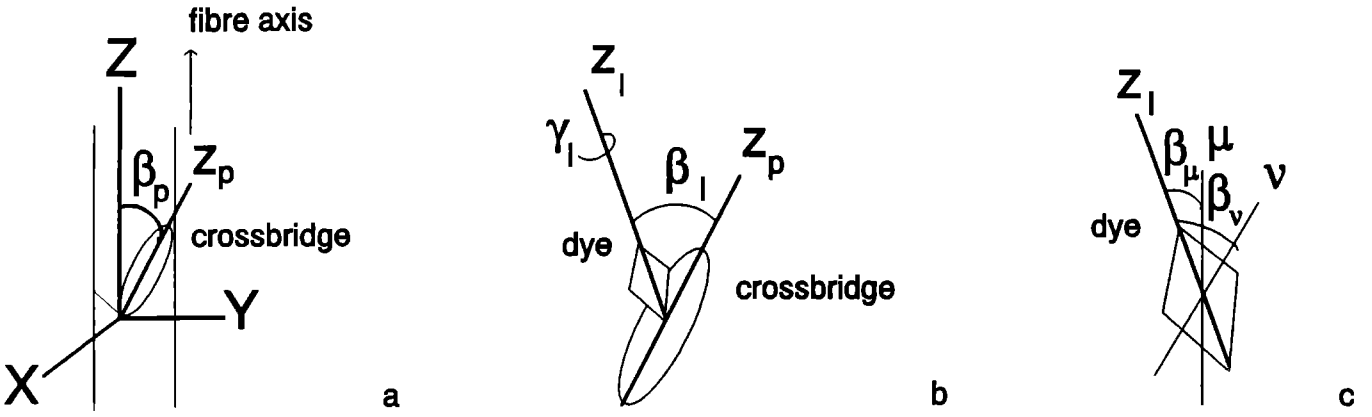


Fig. 1. **a** The orientation of the crossbridge head S_1 relative to the fibre axis. **b** The orientation of a planar dye label relative to S_1. **c** The orientation of the absorption dipole μ and the emission dipole ν in the frame of the dye molecule

rectly. The depolarization effects arise from the changes in the orientation of the transition dipoles of the dye molecules attached to the crossbridge. This implies that the orientation of the transition moments relative to the crossbridge has to be taken into consideration in the analysis of the observations. In this context it is important to note that the observed depolarization is determined by three independent factors: 1) the orientation and rotation of the crossbridges in the muscle fibre, 2) the orientation of the dye molecule relative to the crossbridge and 3) the orientation of the excitation and emission transition moments in the molecular frame of the dye (Fig. 1). It is clear that many parameters now enter the analysis of the experimental data in terms of a molecular model. In order to determine these parameters many independent depolarization ratios need to be measured.

In the most commonly used steady state depolarization experiments however, a fixed scattering geometry is used. This allows the determination of four intensities, i.e. excitation with horizontally and vertically polarized light and detection of horizontally and vertically polarized emission. Therefore only three independent polarization ratios can be determined with this experimental arrangement (Wilson and Mendelson 1983). Consequently this experiment yields insufficient information to disentangle the three contributions to the depolarization process. One approach to circumvent this limitation is to consider only the orientation of the transition moments relative to the fibre-axis (Borejdo and Putnam 1977; Wilson and Mendelson 1983). The advantage of this approach is the reduction in the number of independent parameters entering the analysis, so that information about changes in the depolarization ratios can be correlated with changes in the physiological conditions of the muscle fibres. Nevertheless, it has already been recognized (Ajtaj and Burghardt 1989) that changes in the orientation of the crossbridges fail to result in large changes in the depolarization ratios if the transition moments of the dyes lie in an 'unfavourable plane' in the frame of the crossbridge. This indicates that the results suffer from an explicit dependence on the particular dye and transition moments used in the experiments. Therefore the comparison of results obtained with different dyes or with the same dye at different excitation and emission wavelengths may be hazardous.

The same ambiguities also apply to the analysis of time resolved anisotropy measurements. Here the extraction of the dynamics of the crossbridge rotations again requires knowledge of the orientation of the dye relative to the crossbridge and the orientation of the transition moments in the frame of the dye (Van Gurp et al. 1988 b).

The difficulties in the analysis of depolarization experiments can be overcome if the missing information about the orientation of the transition moments in the frame of the dye, as well as the orientation of the dye relative to the crossbridge can be determined in separate experiments. For this reason we have determined the orientations of fluorescent dyes relative to myosin S_1 (Van der Heide et al. 1992). The latter study also revealed that the absorption, emission and anisotropy spectra of 1,5-I-AEDANS and E5M do not change on binding to the myosin S_1. This indicates that the orientations of the transition dipole moments deduced from experiments on the dyes in solution and in solid matrices remain valid for molecules bound to the protein.

In this study we focus on the determination of the orientations of the absorption and emission transition moments of 1,5-I-AEDANS and E5M, two dyes which are most commonly used in muscle research. To this end the dyes were macroscopically aligned in a poly vinyl alcohol (PVA) film and their steady-state fluorescence depolarization was studied using the angle-resolved method described in (Van Gurp et al. 1988 a; Van Gurp and Levine 1989 b). Here the depolarization ratios are measured as a function of two independent angles: 1) the angle between the exciting beam and the stretch direction of the film and 2) the angle between direction of the polarized fluorescence emission and the excitation light beam (Fig. 2).

Theory

The orientational distribution of molecules in an ordered host matrix can be described by a probability function $P(\Omega)$. Here Ω denotes the three Euler angles $\{\alpha\ \beta\ \gamma\}$ defin-

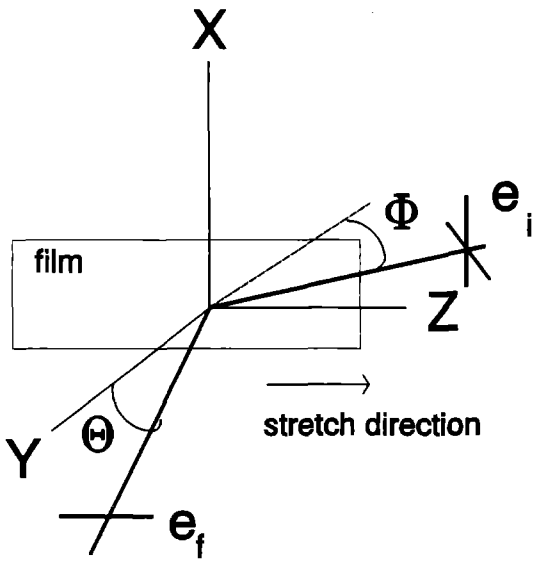


Fig. 2. The geometry of the angle resolved depolarization experiment on stretched polymer films

ing the rotational transformation from the laboratory to the molecular frame. In the following calculations it will prove convenient to expand this distribution function in a series of Wigner functions $D^L_{mn}(\Omega)$, which form an orthonormal set of goniometric functions. The Wigner functions represent the transformation between two coordinate systems (Rose 1957). Now:

$$P(\Omega) = \sum_{L\,mn} \langle D^{L*}_{mn}(\Omega) \rangle D^L_{mn}(\Omega) \tag{1}$$

with:

$$\langle D^{L*}_{mn}(\Omega) \rangle = \int d\Omega\, D^{L*}_{mn}(\Omega)\, P(\Omega) \tag{2}$$

The moments $\langle D^{L*}_{mn} \rangle$ of the series expansion are called order parameters.

We shall here only consider dipole-allowed excitation and emission transitions of the dye molecules. The intensity of fluorescence emission following the application of a narrow pulse of excitation light can be written as:

$$I_{if}(t) \propto \int d\Omega\, (\boldsymbol{e}_f \cdot \nu)^2\, (\boldsymbol{e}_i \cdot \mu)^2\, P(\Omega)\, F(t) \tag{3}$$

with

I_{if} the detected fluorescence intensity
$\boldsymbol{e}_i$ the polarization vector of the exciting beam
$\boldsymbol{e}_f$ the polarization vector of the detected fluorescence
μ the absorption dipole moment of the dye
ν the emission dipole moment of the dye
$F(t)$ the fluorescence life time function
$P(\Omega)$ the orientational distribution function of the dye.

The scalar products can be expressed in terms of second order Legendre polynomials with the cosine of the angle between the two vectors as an argument:

$$(\boldsymbol{e}_i \cdot \mu)^2 = \frac{2P_2(\cos(\boldsymbol{e}_i \cdot \mu)) + 1}{3} \tag{4}$$

and

$$(\boldsymbol{e}_f \cdot \nu)^2 = \frac{2P_2(\cos(\boldsymbol{e}_f \cdot \nu)) + 1}{3} \tag{5}$$

with

P_2 the second order Legendre polynomial.

Equations (4) and (5) can now be separated into factors describing the molecular properties and geometrical effects on making use of the closure relation of the Wigner functions (Rose 1957; Zannoni 1979). This involves a representation of the polarization vectors and the transition moments in the frame of the sample. Thus:

$$P_2(\cos(\boldsymbol{e}_i \cdot \mu)) = D^2_{00}(-\Omega_{e_i} + \Omega_\mu) = \sum_{i=-2}^{2} D^{2*}_{i0}(\Omega_{e_i})\, D^2_{i0}(\Omega_\mu) \tag{6}$$

and

$$P_2(\cos(\boldsymbol{e}_f \cdot \nu)) = D^2_{00}(-\Omega_{e_f} + \Omega_\nu) = \sum_{j=-2}^{2} D^2_{j0}(\Omega_{e_f})\, D^{2*}_{j0}(\Omega_\nu) \tag{7}$$

with

$D^L_{mn}(\Omega)$ the Wigner functions (Rose 1957)
Ω_{e_i} the Euler angles of the polarization vector of the excitation beam in the sample frame
Ω_{e_f} the Euler angles of the polarization vector of the observed fluorescence emission in the sample frame
Ω_μ the Euler angles of the absorption dipole of the dye molecule in the sample frame
Ω_ν the Euler angles of the emission dipole of the dye molecule in the sample frame.

After some laborious, but straightforward algebra, (3) is reduced using (4)–(7) to

$$I_{if}(t) \propto \left(1 + 2D^2_{00}(\Omega_{e_i})\, S_\mu + 2D^2_{00}(\Omega_{e_f})\, S_\nu + 4 \sum_{k=-2}^{2} D^{2*}_{k0}(\Omega_{e_i})\, D^2_{k0}(\Omega_{e_f})\, G_k\right) F(t) \tag{8}$$

with

$$S_\mu = \langle D^2_{00}(\Omega_\mu) \rangle = \langle P_2(\cos\beta_\mu) \rangle$$
$$S_\nu = \langle D^2_{00}(\Omega_\nu) \rangle = \langle P_2(\cos\beta_\nu) \rangle$$
$$G_k = \langle D^2_{k0}(\Omega_\mu)\, D^{2*}_{k0}(\Omega_\nu) \rangle \qquad k = 0, 1, 2$$

In deriving (8) we assumed the distribution of the transition moments to be cylindrically symmetric in the sample frame, so that all terms $\langle D_{i0}(\Omega_\mu) \rangle$, $\langle D_{j0}(\Omega_\nu) \rangle$ vanish for $i, j \neq 0$. S_μ and S_ν are commonly known as the order parameters of the absorption and emission moment respectively. The terms G_k, are rotational correlation functions, and for a cylindrically symmetric distribution $G_k = G_{-k}$. Consequently at most five parameters can be determined in a depolarization experiment irrespective of its geometry.

Fluorescence depolarization of labelled crossbridges in a muscle fibre

We shall now illustrate this theoretical approach with a description of a fluorescence depolarization experiment on labelled muscle fibres aligned along the z-axis of a laboratory frame. On using a 90° scattering geometry four independent intensities can be determined: I_{vv}, I_{vh}, I_{hv} and I_{hh}, where the first index denotes the direction of

the excitation polarizer, vertical or horizontal, and the second index denotes the direction of the emission polarizer. These intensities can be derived from (8) as:

$$\begin{aligned} I_{vv} &\propto (1+2\,S_\mu+2\,S_\nu+4\,G_0) \\ I_{vh} &\propto (1+2\,S_\mu-S_\nu-2\,G_0) \\ I_{hv} &\propto (1-S_\mu+2\,S_\nu-2\,G_0) \\ I_{hh} &\propto (1-S_\mu-S_\nu+G_0-3\,G_2) \end{aligned} \tag{9}$$

A description of these intensities in cartesian coordinates has been given by (Wilson and Mendelson 1983).

As noted previously (Van Gurp et al. 1988 b), the correlation function G_1 cannot be determined experimentally using either a 90° or a 180° scattering geometry. It can be seen from (9) that only 3 independent parameters can be obtained from the experimental data. Nevertheless, all 5 independent parameters can be determined using angle-resolved fluorescence depolarization techniques.

The accessible information about the orientation of muscle crossbridges relative to the fibre must now be extracted from the 5 experimental parameters, S_μ, S_ν, G_0, G_1 and G_2. Following the same approach used above (3) can be expressed using 3 successive rotational transformations (Fig. 1): 1) from the frame of the fibre to the frame of the crossbridge, 2) from the frame of the crossbridge to the frame of the dye molecule and 3) from the molecular frame to the excitation and emission transition moments. It can be shown that the 5 parameters can be expressed as:

$$\begin{aligned} S_\mu &= \sum_{j=-2}^{2} \langle D^2_{00}(\Omega_p)\rangle\, D^2_{0j}(\Omega_l)\, D^2_{j0}(\Omega_\mu) \\ S_\nu &= \sum_{r=-2}^{2} \langle D^2_{00}(\Omega_p)\rangle\, D^{2*}_{0r}(\Omega_l)\, D^{2*}_{r0}(\Omega_\nu) \\ G_k &= \sum_{i,j,r=-2}^{2} \langle D^2_{ki}(\Omega_p)\, D^{2*}_{ki}(\Omega_p)\rangle\, D^2_{ij}(\Omega_l)\, D^{2*}_{ir}(\Omega_l) \\ &\quad \cdot D^2_{j0}(\Omega_\mu)\, D^{2*}_{r0}(\Omega_\nu) \qquad k=0,1,2 \end{aligned} \tag{10}$$

with

Ω_p the Euler angles of the crossbridge in the fibre frame
Ω_l the Euler angles of the dye in the frame of the crossbridge
Ω_μ the orientation of the absorption moment in the frame of the dye
Ω_ν the orientation of the emission moment in the frame of the dye.

In deriving these equations we have made the following assumptions:

- The absorption and fluorescence emission moments of dyes such as 1,5-I-AEDANS and E5M were taken to lie in the plane of these molecules. Therefore only the Euler angles β_μ, β_ν of the transition moments relative to the long axis of the dye enter the equations as the angles α_μ and α_ν may be taken to be 0.
- The orientational distribution of crossbridges relative to the fibre is invariant for rotation around the fibre axis (Z-axis).
- The fibre possesses a mirror symmetry in the X-Y plane as a result of opposite half sarcomeres.
- For the sake of simplicity we have taken the dyes to have one fixed orientation relative to the crossbridge and to exhibit no motion on the time scale of the experiment. While there is evidence from time resolved experiments for relative motion between the dye and the crossbridge, (Van der Meulen et al. 1990, Van der Heide et al. 1992) this motion appears to be restricted.

The orientational distribution function of the crossbridges relative to the axis of the muscle fibre is fully characterized only if all the order parameters $\langle D^L_{00}(\Omega_p)\rangle$ are known. However, it can be seen from (10) that only the lowest rank parameters, $L=2$ and 4, are accessible experimentally. The difficulty now is the extraction of the order parameters from the label and transition moment dependent parameters S_μ, S_ν, G_0, G_1 and G_2. This can only be achieved if the orientation of the transition moments in the frame of the dye as well as the orientation of the dye molecule in the crossbridge frame are known. In this paper we shall only address the former question, the determination of the orientation of the transition moments in the molecular frame.

Determination of the orientation of transition moments

Dye molecules can be aligned macroscopically in a stretched polymer matrix, so that they do not undergo rotational motions on the timescale of the fluorescence life time. The orientation of the transition moments in the molecular frame can now be determined with the technique of Angle resolved Fluorescence Depolarization, as described in (Van Gurp et al. 1988 a; Van Gurp and Levine 1989 b). Here we shall only consider the interpretation of the 5 experimentally accessible parameters, S_μ, S_ν, G_0, G_1 and G_2 (Fig. 3). Now:

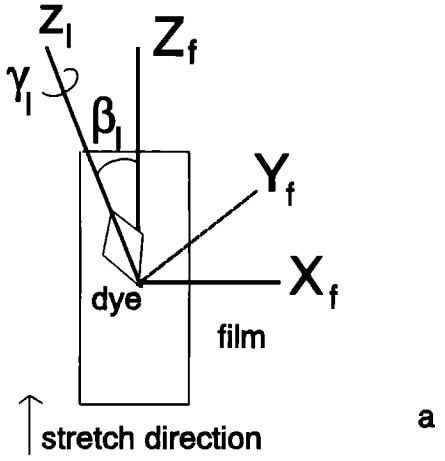
$$\begin{aligned} S_\mu &= \sum_{i=-2}^{2} \langle D^2_{0i}(\Omega_l)\rangle\, D^2_{i0}(\Omega_\mu) \\ S_\nu &= \sum_{j=-2}^{2} \langle D^2_{0j}(\Omega_l)\rangle\, D^2_{j0}(\Omega_\nu) \\ G_k &= \sum_{i,j=-2}^{2} \langle D^2_{ki}(\Omega_l)\, D^{2*}_{kj}(\Omega_l)\rangle\, D^2_{i0}(\Omega_\mu)\, D^{2*}_{j0}(\Omega_\nu) \quad k=0,1,2 \end{aligned} \tag{11}$$

with

Ω_l the orientation of the dye molecules relative to the stretch direction of the film
Ω_μ the orientation of the absorption moment in the frame of the dye
Ω_ν the orientation of the emission moment in the frame of the dye.

In deriving (11) we assume the following symmetry relations to hold (Van Gurp et al. 1988 a; Van Gurp and Levine 1989 b, Van Gurp and Levine 1991):

- The distribution function of the dye is invariant under a rotation around the stretching direction of the film (Z-axis). This implies that all α-dependent order parameters vanish so that: $\langle D^L_{mn}(\Omega_l)\rangle = \langle D^L_{mn}(\Omega_l)\rangle\, \delta_{m0}$



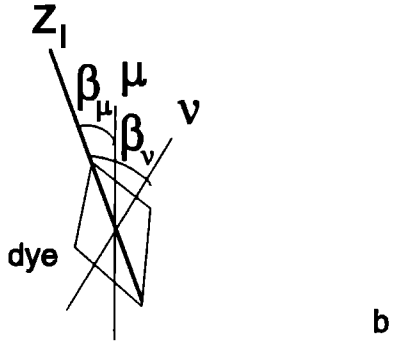


Fig. 3. a The orientation of a planar dye in the frame of the stretched polymer film. **b** The orientation of the absorption dipole μ and the emission dipole ν in the frame of the dye molecule

- The distribution function of the dyes possesses mirror symmetry in the XY- and XZ-planes of the film. Consequently $\langle D^L_{mn}(\Omega_l)\rangle = (-1)^L \langle D^L_{mn}(\Omega_l)\rangle$ showing that all order parameters with odd L vanish.
- The distribution of the dye molecules is invariant to reflections in the xz- and yz-planes of the planar dye molecule. This double reflection symmetry leads to the following relations: $\langle D^L_{mn}(\Omega_l)\rangle = (-1)^n \langle D^L_{mn}(\Omega_l)\rangle$ and $\langle D^L_{mn}(\Omega_l)\rangle = (-1)^{L-m} \langle D^L_{m-n}(\Omega_l)\rangle$.

Equation (11) now shows that only five non-vanishing independent order parameters determine the measured intensities:

$$\langle P_2\rangle, \langle D^2_{02}\rangle, \langle P_4\rangle, \langle D^4_{02}\rangle, \langle D^4_{04}\rangle .$$

This is a direct consequence of the fact that only dipole-allowed transitions are considered because of the negligible contribution of multipole radiation to the fluorescence process. This limitation is an inherent property of fluorescence depolarization techniques (Birks 1970).

In view of the limited number of experimentally accessible order parameters, we reconstruct the orientational distribution function using the Maximum Entropy Method. This approach yields the broadest possible distribution function that is consistent with the known order parameters (Van Gurp et al. 1988 b). In order to limit the number of fit parameters we have only included the three most significant Wigner functions in the distribution function. This choice is justified by our expectation of a smooth distribution of dye molecules in the polymer matrix. The orientational distribution function is now given by:

$$P(\Omega_l) = A\, e^{-(\lambda_2 P_2(\cos\beta_l) + \lambda_4 P_4(\cos\beta_l) + \varepsilon(D^2_{02}(0\,\beta_l\gamma_l) + D^2_{0-2}(0\,\beta_l\gamma_l)))} \quad (12)$$

with

A a normalization constant
$\lambda_2, \lambda_4, \varepsilon$ the parameters describing the strength of the potential.

Now the 5 parameters appearing in (8) can be expressed in terms of 3 parameters for the orientational distribution of the dye molecules in the film and 2 parameters for the orientation of the transition moments in the plane of the dye. This means that the orientation of transition moments in a dye can be obtained following this approach.

In the preceding theory we implicitly assumed that the fluorescence and absorption processes arise from pure electronic transitions. This however is not generally true as it is possible to excite more than one electronic transition at a given wavelength owing to vibrational broadening of the absorption bands. Furthermore, the excited electronic states of the molecules may also be mixed by vibronic interactions. We have previously shown that in this case the parameters $D^2_{i0}(\Omega_\mu)$ and $D^2_{j0}(\Omega_\nu)$ in the theory above may be replaced by the weighted average of the transition moments involved (Van Gurp et al. 1989 a).

Materials and methods

Sample preparation

Eosin-5-Maleimide and 1,5-I-AEDANS were obtained from Molecular Probes. N-acetylcysteine was obtained from Sigma Chemical Company. Polyvinyl alcohol (PVA) with an average molecular weight of 115 kDa was purchased from Aldrich Chem. Company.

Eosin-5-Maleimide was used without further purification or modification. Photodegradation of 1,5-I-AEDANS was prevented by reacting the dye with N-acetylcysteine to form the stable 1,5-AcCys-AEDANS, following the method described in Hudson and Weber (1973).

The dye molecules were incorporated in stretched PVA-films using a modification of the method described earlier (Van Gurp et al. 1988 a, b). 1.5 gram of PVA powder was added to 15 ml of water. The mixture was heated slowly to 80 °C under continuous stirring and was kept at this temperature until a clear solution was obtained. It was then cooled down slowly to 40 °C, when a dye solution was added up to a final concentration of 15 μM. Solid films were cast by pouring 1 ml of the solution on to a glass plate and drying for 2 days. After this, the films were kept for 24 hours in a desiccator with a relative humidity of 96%. Subsequently, they were stretched at room temperature up to 4 times their original length. These stretched films were pressed between quartz plates ($n = 1.46$) using glycerol (spectro-photometric grade,

Aldrich Chemical Company, $n=1.4716$) to improve optical contact. Finally, the samples were sealed at the edges with glue.

The refractive index of the PVA-films was taken to be $n=1.52$. The slight birefringence of the films ($n_e=1.537$ and $n_0=1.511$) had no material effect on the results obtained (Van Gurp et al. 1988a).

Angle-resolved Fluorescence Depolarization

Angle-resolved fluorescence depolarization (AFD) measurements were carried out on a home built set-up as described in Van Gurp et al. (1988b). A 1 600 W Xenon arc lamp (OSRAM) was used as light source. The light was focused on the entrance slit of a Jarell-Ash monochromator with a quartz lens. The light then passed through a combination of a chopper, interference filters and a quartz lens in order to obtain a narrow-band parallel beam. This beam was polarized in a horizontal direction with a Glan Taylor prism before incidence on the sample. The stretched film was mounted on a rotating stage with its plane vertical and the stretch direction horizontal. This allowed the angle between the stretch direction and the excitation beam to be varied. The fluorescence emission was detected at various angles relative to the exciting beam by rotating an optical rail, on which a lens, a sheet polarizer, a set of filters and a Philips XP2020Q photomultiplier tube were mounted, around the sample. The output signal of the PMT was fed into a pre-amplifier before entering a Lock-in amplifier (Princeton Applied Research 124A). Data was acquired in digital form using a 12-bits ADC interfaced to a personal computer.

The wavelength of the incident light was varied in order to excite different absorption transition moments of each molecule. Scattered light was rejected by mounting a combination of interference filters and cut-off filters in front of the PMT. In this way the emission from a single transition was detected by selecting a narrow-band around the maximum of the spectrum.

Results

Depolarization ratios were measured at up to 56 different combinations of the angles of incidence, θ, and detection, Φ (Fig. 2). The data were fitted directly to the molecular parameters rather than the 5 experimental quantities S_μ, S_ν, G_0, G_1 and G_2. The non-linear least squares Marquart procedure (ZXSSQ) from the IMSL library was used for the optimization of the parameter values using a Global Target approach (Arcioni et al. 1988). Data sets collected at different excitation wavelengths were analyzed simultaneously in order to satisfy the requirement of overdetermination for least squares fitting. Data sets obtained from experiments on a single sample using the same emission wavelength were used in the analysis. This stratagem reduced the number of free model parameters since the same orientational distribution factor enters the description of every data set. Moreover, the orientation of the

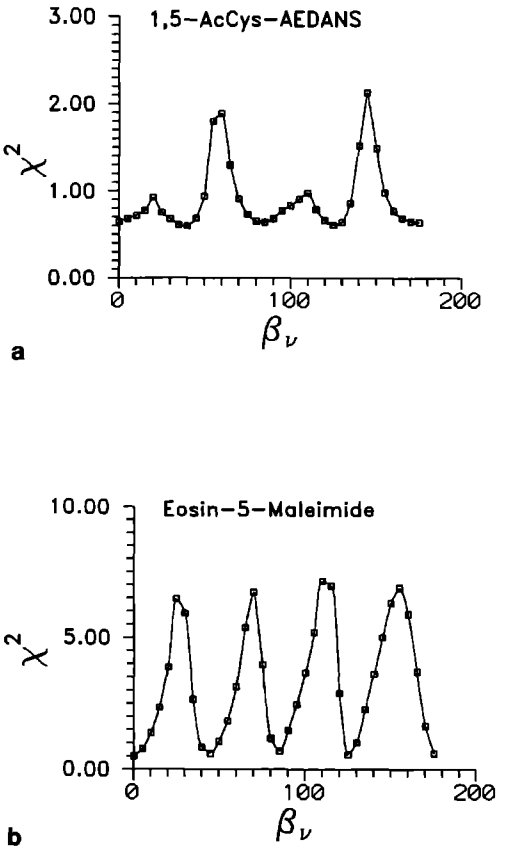


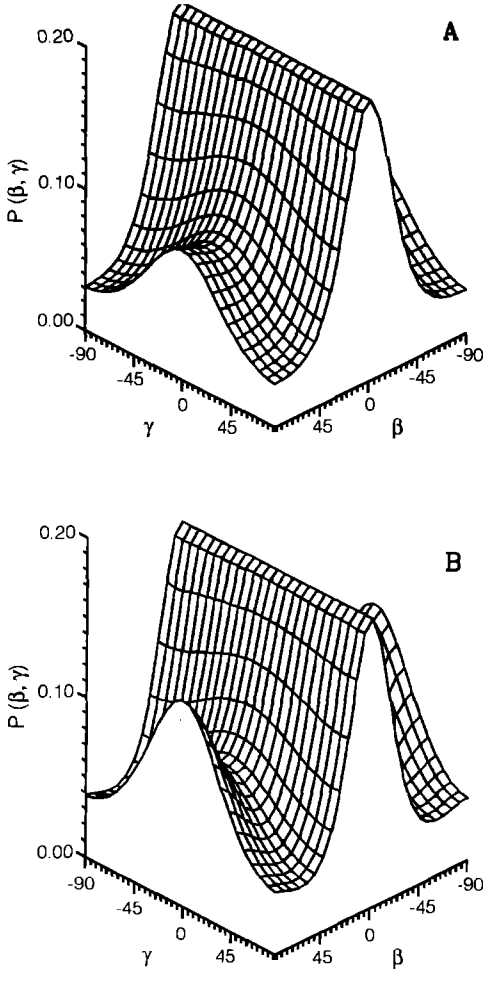
Fig. 4. The minimal value of χ^2 at fixed values of β_ν. **a** 1,5-AcCys-AEDANS, **b** eosin-5-maleimide

Table 1. The orientational distribution functions and the corresponding orientations of transition moments of 1,5-AcCys-AEDANS

	Solution A	Solution B
Potential parameters		
λ_2	0.74 ±0.03	0.37 ±0.18
λ_4	0.335±0.015	0.62 ±0.05
ε	0.9 ±0.2	1.00 ±0.15
Order parameters		
$\langle P_2 \rangle$	0.145±0.006	0.06 ±0.03
$\langle D^2_{02} \rangle$	0.11 ±0.02	0.143±0.008
$\langle P_4 \rangle$	0.063±0.002	0.084±0.002
$\langle D^4_{02} \rangle$	0.009±0.002	0.008±0.006
$\langle D^4_{04} \rangle$	0.016±0.006	0.023±0.004
λ_ν [nm]	β_ν	β_ν
464	177±5°	86±5°
λ_μ	β_μ	β_μ
320	32±5°	120±5°
330	35±5°	124±5°
342	32±5°	121±5°
350	26±5°	115±5°
362	20±5°	109±5°
370	18±5°	107±5°
390	15±5°	105±5°

Table 2. The orientational distribution functions and the corresponding orientations of transition moments in eosin-5-maleimide

	Solution A	Solution B	Solution C	Solution D
Potential parameters				
λ_2	-0.11 ± 0.03	2.3 ± 0.3	0.1 ± 0.5	0.65 ± 0.02
λ_4	0.899 ± 0.003	-2.29 ± 0.03	1.44 ± 0.08	-2.2 ± 0.7
ε	0.46 ± 0.40	-0.29 ± 0.05	0.1 ± 0.2	-0.068 ± 0.016
Order parameters				
$\langle P_2 \rangle$	-0.023 ± 0.015	-0.09 ± 0.5	0.06 ± 0.14	0.111 ± 0.003
$\langle D^2_{02} \rangle$	0.08 ± 0.07	0.050 ± 0.011	0.01 ± 0.02	-0.008 ± 0.001
$\langle P_4 \rangle$	0.104 ± 0.002	-0.147 ± 0.017	0.18 ± 0.02	-0.206 ± 0.06
$\langle D^4_{02} \rangle$	-0.012 ± 0.001	0.008 ± 0.001	-0.003 ± 0.001	-0.005 ± 0.002
$\langle D^4_{04} \rangle$	0.006 ± 0.006	0.002 ± 0.001	0.000 ± 0.002	0.000 ± 0.001
λ_ν [nm]	β_ν	β_ν	β_ν	β_ν
547	$2 \pm 5°$	$44 \pm 5°$	$84 \pm 5°$	$126 \pm 5°$
λ_μ	β_μ	β_μ	β_μ	β_μ
325	$64 \pm 5°$	$114 \pm 5°$	$147 \pm 5°$	$15 \pm 5°$
330	$62 \pm 5°$	$111 \pm 5°$	$145 \pm 5°$	$13 \pm 5°$
345	$64 \pm 5°$	$114 \pm 5°$	$147 \pm 5°$	$15 \pm 5°$
375	$63 \pm 5°$	$111 \pm 5°$	$145 \pm 5°$	$13 \pm 5°$
405	$51 \pm 5°$	$100 \pm 5°$	$134 \pm 5°$	$1 \pm 5°$
440	$36 \pm 5°$	$82 \pm 5°$	$119 \pm 5°$	$164 \pm 5°$
470	$24 \pm 5°$	$70 \pm 5°$	$107 \pm 5°$	$151 \pm 5°$
505	$22 \pm 5°$	$67 \pm 5°$	$106 \pm 5°$	$149 \pm 5°$
515	$24 \pm 5°$	$69 \pm 5°$	$108 \pm 5°$	$151 \pm 5°$

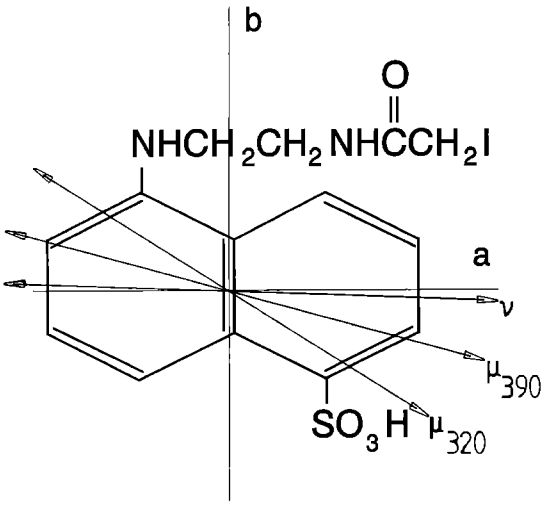


emission dipole in the frame of the dye molecule is constant within the group of data sets. The difference in the polarization ratios in the data sets arises solely from differences in the orientation of the absorption transition moments in the dye molecule.

This approach now requires the numerical optimization of $N+4$ independent parameters to fit a total number of N data sets each consisting of 50–56 polarization ratios. In this way a sharper χ^2-surface was found compared to that obtained from a fit to a single data set. Nevertheless, the global χ^2-surface exhibited a number of equivalent minima (Fig. 4). The value of the global χ^2 was not significantly higher than the average of the χ^2's obtained from fits to the individual data sets. This indicates that the orienting potential and the orientation of the emission moment are indeed independent of the excitation wavelength.

It is important to note that the degeneracies observed in the fitting procedure are inherent in the description of the experiment. The molecular symmetry of the dye admits different choices for the z-axis of its frame. Each choice requires a distinct orientational distribution of the molecular frame in the film in order to reproduce the same physical situation. Consequently the analysis will yield a number of physically equivalent, but numerically distinct solutions. The number of numerically distinct solutions is limited by the symmetry of the molecular orientational distribution used in the analysis.

Fig. 5. The orientational distribution functions of 1,5-AcCys-AEDANS in the film, corresponding to the potentials in Table 1



The parameters obtained from the best fits are shown in Tables 1 and 2. It is important to realize that the orientational distribution functions and their corresponding order parameters are specific to the sample studied. In contrast, every sample yielded the same orientations of the transition moments in the molecular frame of the dye molecule. The relative orientations of the absorption and emission transition moments extracted from the analysis are found to be consistent with literature values for the fluorescence anisotropy determined in viscous, isotropic solutions of the dyes (Hudson and Weber 1973, Vander-Meulen et al. 1990).

Fig. 6. The orientations of the transition moments in 1,5-AcCys-AEDANS

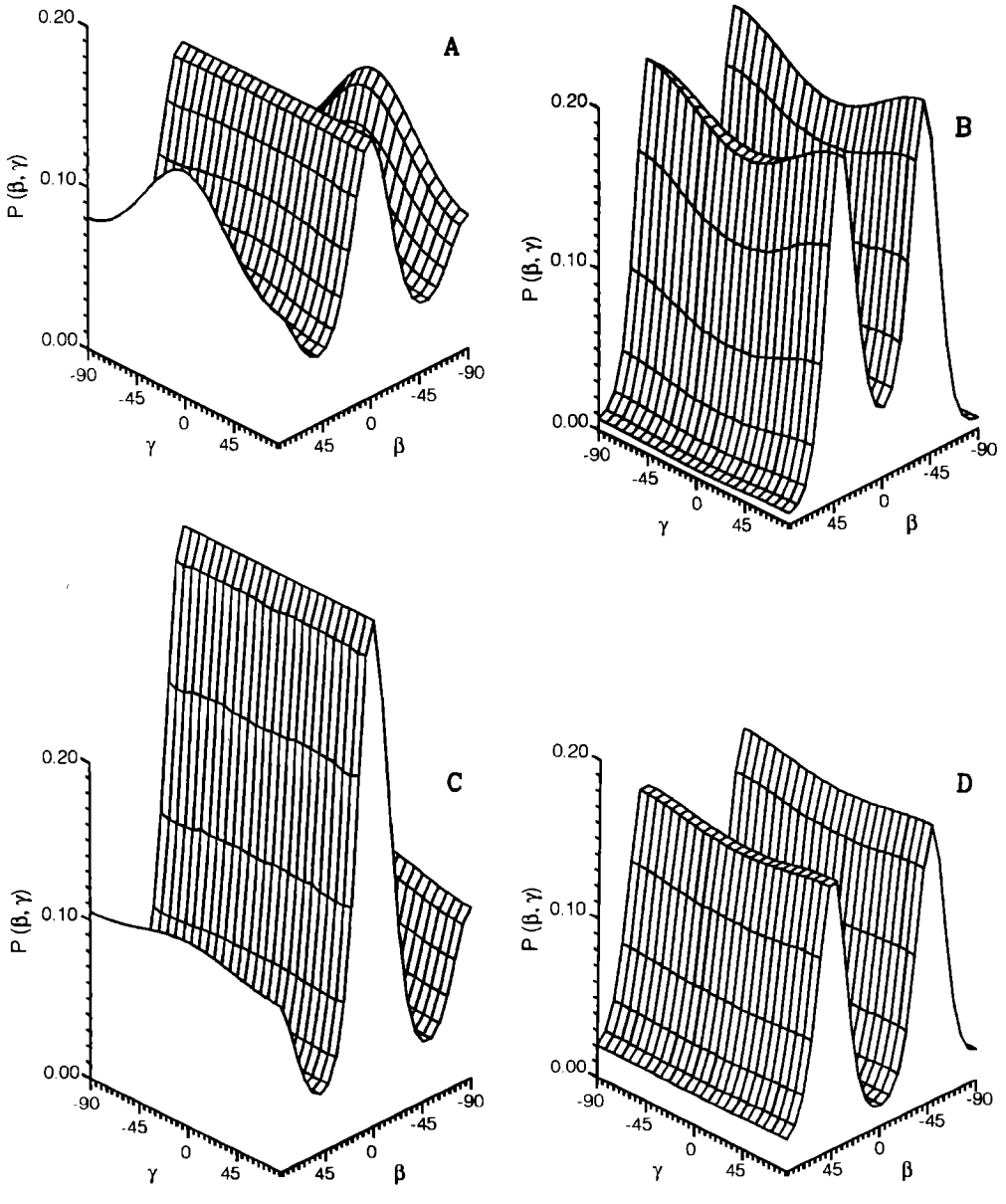


Fig. 7. The orientational distribution functions of eosin-5-maleimide in the film, corresponding to the potentials in Table 2

The assumption of pure electronic transitions underlying our analysis was tested by considering the case of the simultaneous excitation of two orthogonal absorption moments. We have here taken the two moments to lie along the z- and x-axis of the molecular frame. Now, the parameter optimized by the least-squares procedure is the relative contribution of each transition to the observed fluorescence at different wavelengths. This approach, however, failed to decrease the values of χ^2 found using the analysis above. We therefore believe that the use of an effective orientation of impure transition moments provides an adequate description of fluorescence depolarization experiments.

It is reasonable to expect molecules such as 1,5-AcCys-AEDANS and E5M to be preferentially oriented with their longer axis parallel to the stretch direction of the polymer film. Nevertheless, a smaller fraction will be oriented with its shorter axis parallel to that direction. However, it is unlikely that a dye molecule will be oriented with its plane perpendicular to the stretch direction.

The results for 1,5-AcCys-AEDANS in PVA yielded two numerically distinct but statistically equivalent solutions A and B as given in Table 1. The corresponding orientational distributions are shown in Fig. 5. The global maximum of the distribution function corresponding to solution A is found at $\beta_l = 0°$ independent of the angle γ_l. Here the molecular z-axis is aligned parallel to the stretch direction of the film. The local maxima found at $\beta_l = 90°$ and $\gamma_l = 0°$ correspond to dye molecules oriented with their z-axes perpendicular to the stretch direction but with the in-plane x-axes parallel to this direction. The distribution corresponding to solution B has a similar shape, but the local maximum at $\beta_l = 90°$ and $\gamma_l = 0°$ is now significantly larger than that found in solution A. It has to be noted that the distribution functions are shown as a function of the Euler angles β and γ. The fraction of molecules lying at orientations between $\{\beta, \gamma\}$ and $\{\beta + \Delta\beta, \gamma + \Delta\gamma\}$ is now given by $\sin\beta\, P(\beta, \gamma)\, \Delta\beta\, \Delta\gamma$. As noted above, each solution corresponds to a different choice for the z-axis in the molecule. We therefore identify the z-axis in solution A with the long axis of the dye molecule and the z-axis in solution B with its shorter axis.

The orientations of the excitation moments at 330 and 370 nm and the emission moment in the molecule are shown schematically in Fig. 6. It can be seen easily from the figure that the two solutions A and B describe the same physical situation.

The depolarization data for E5M in PVA yield four equivalent solutions as a result of the symmetry of the molecule, Table 2. The distribution functions corresponding to these solutions are shown in Fig. 7. On following the reasoning set out above for 1,5-AcCys-AEDANS, we conclude that the z-axis of the dye in solution C is the longer axis of the molecule. The z-axis of solution A is identified with the shorter in-plane axis. The two solutions B and D are consistent with this conclusion. In these two situations the orientational distributions show that the z-axes are preferentially tilted relative to the stretch direction of the film. This implies that the z-axes are now chosen so as to lie along the diagonals of the molecular structure so that the orientational distribution is independent of γ. The orientation of the emission and absorption moments at 330 and 505 nm is shown schematically in Fig. 8.

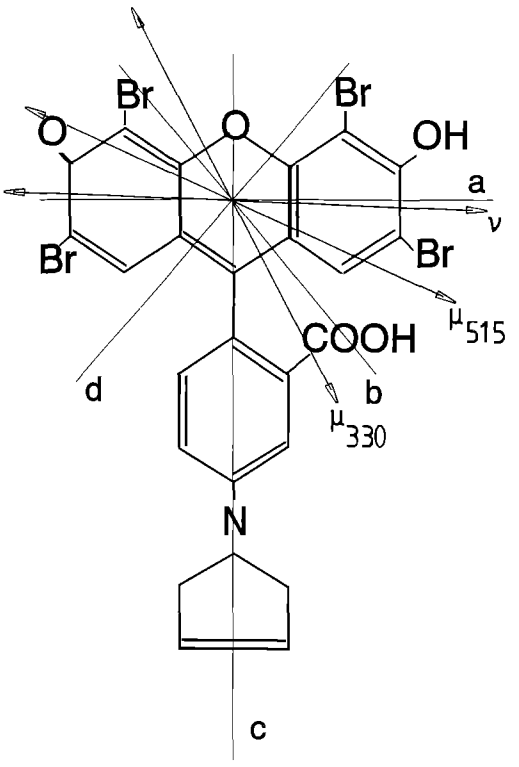


Fig. 8. The orientations of the transition moments in eosin-5-maleimide

Conclusion

We have here shown that the probe-independent interpretation of fluorescence depolarization experiments on dye labelled muscle fibres calls for information on the orientation of transition moments in the dye molecule. In addition, the orientation of a dye relative to the crossbridge must be known. It is not possible to extract these orientations from a single depolarization experiment on muscle fibres. However, it cannot be ruled out that the necessary information could be extracted from a combination of experimental data obtained with several dyes exciting at a variety of different absorption wavelengths.

We have chosen to characterize the orientation of the transition moments in separate experiments prior to using this information in the interpretation of fluorescence depolarization experiments of labelled muscle fibres. These orientations can be obtained from angle-resolved fluorescence depolarization experiments on dyes macroscopically aligned in a stretched polymer matrix. The robustness of this approach is enhanced by a global target analysis of data obtained at different excitation wavelengths. The orientations of the transition moments found in this way are consistent with the angles between absorption and emission moments derived from fluorescence anisotropy measurements.

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