Separation of the rotational contribution in fluorescence correlation experiments

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ABSTRACT The theory of fluorescence correlation spectroscopy is reexamined with the aim of separating the contribution of rotational diffusion. Under constant excitation, fluorescence correlation experiments are characterized by three polarizations: one of the incident beam and two of the two photon detectors. A set of experiments of different polarizations is proposed for study. From the results of the experiments the isotropic factor of the fluorescence intensity correlation functions can be determined, which is independent of the rotational motion of the sample molecule. This function can be used to represent each fluorescence intensity correlation function as the product of the isotropic and the rotational factors. The theory is illustrated by an experiment in which rotational diffusion of porcine pancreatic lipase labeled with Texas Red was observed. Texas Red is a label that allows precise fluorescence correlation experiments even in the nanosecond time range.

INTRODUCTION

In fluorescence correlation spectroscopy (FCS) the behavior of individual fluorescent molecules is studied (Elson and Magde, 1974). Most applications of FCS are related to translational motion: the detected fluorescence intensity of a molecule changes as the molecule enters or leaves the sample domain. Translational diffusion of labeled material in various environments is often under direct scrutiny (Magde et al., 1974; Schlessinger et al., 1976; Webb, 1976; Sorscher et al., 1980; Andries et al., 1983; etc.). Translational diffusion is sometimes used as a tool to study binding reactions restricting diffusion (Borejdo, 1979; Thompson and Axelrod, 1983). Scanning as a peculiar type of translational motion has been used to study the extent of polymerization or aggregation (Weissman et al., 1976; Petersen, 1984; Palmer and Thompson, 1987).

In addition to translational motion, a number of processes are known to be responsible for fluorescence intensity fluctuations: rotational motion (Ehrenberg and Rigler, 1974), conformational changes (Steinberg and Haas, 1982), spontaneous and photoinduced chemical reactions (Elson and Magde, 1974), and some photophysical processes (Ehrenberg and Rigler, 1974; Kändler et al., 1982; Kask et al., 1985).

Several theoretical papers have been published on FCS applications to rotational motion (Ehrenberg and Rigler, 1974, 1976; Aragon and Pecora, 1975, 1976; Yardley and Specht, 1976; Hoshikawa and Asai, 1985). Despite the fact that important information can be derived from the FCS measurements in the wide range of rotational correlation times, from 10^{-8} to 1 s, relatively few experiments have been followed up (Borejdo et al., 1979; Kask et al., 1987). One reason for this has been the danger of confusing unwanted contributions from the other processes, e.g., from photobleaching or from translational motion.

It is widely known that rotational motion is expressed in various ways and to multiple extents at different polarizations of fluorescence experiments. This property has been used to separate the rotational contribution in fluorescence photobleaching recovery (Wegener and Rigler, 1984) as well as in time-resolved fluorescence depolarization (Tao, 1969).

The first attempt to separate the rotational contribution in FCS was that of Borejdo et al. (1979), who studied fluctuations of the polarization ratio of fluorescence. In the present work the theory of FCS is reexamined with the aim of separating the contribution of rotational diffusion in fluorescence intensity correlation functions. As a model in illustrative experiments, porcine pancreatic lipase labeled with a highly fluorescent and photostable marker Texas Red (Titus et al., 1982) has been chosen.

THEORY

Factorization of the fluorescence intensity correlation function

There exist a multitude of random variables determining the probability density of the sample molecule of absorbing or emitting photons. Let us separate the orientation of the molecule $\Omega(t)$ from all the other variables $\mathbf{Q}(t)$, including the position (in space), chemical state, configu-

ration, and electronic state of the fluorophore molecule. The probability density of absorption is expressed as a function of Ω and Q:

$$W_a(t) = A^2(\Omega(t))F_a(Q(t)). \quad (1)$$

Here $A^2(\Omega)$ depends on the direction of the transition moment of absorption of the molecule, $\hat{\mu}_a(\Omega)$, in respect to the electric vector of the exciting light, $\hat{\mathbf{a}}$ (Ehrenberg and Rigler, 1974):

$$A^2(\hat{\mu}_a(\Omega)) = 3(\hat{\mu}_a \cdot \hat{\mathbf{a}})^2. \quad (2)$$

Similarly to Eqs. 1 and 2, the expressions for the detection of the light emission look like

$$W_e(t) = E^2(\Omega(t))F_e(Q(t)), \quad (3)$$

$$E^2(\hat{\mu}_e(\Omega)) = 3(\hat{\mu}_e \cdot \hat{\mathbf{e}})^2. \quad (4)$$

Here $\hat{\mu}_e$ is the unit vector of the transition moment of the emission of the molecule, and $\hat{\mathbf{e}}$ is the direction of the polarization of the light detector.

Let us imagine an experiment in which fluorescence of a molecule is exited by two short light pulses at the time moments t_1 and t_3 ($t_1 < t_3$), and the joint probability density to detect fluorescence photons at t_2 and t_4 is measured. (Of course, to estimate the probabilities, the experiment has to be repeated very many times.) The measured function is a fourth-order correlation function $G^{(4)}(t_1, t_2, t_3, t_4)$, useful to be studied in the FCS theory (Aragon and Pecora, 1975), although only the second-order fluorescence intensity correlation function under constant excitation is usually measured in experiments.

Using expressions 1 and 3, the fourth-order correlation function is expressed as

$$G^{(4)}(t_1,t_2,t_3,t_4) = \langle A^2(\Omega_1)F_a(Q_1)E^2(\Omega_2)F_e(Q_2) \cdot A^2(\Omega_3)F_a(Q_3)E^2(\Omega_4)F_e(Q_4)\rangle. \quad (5)$$

Assume now that Ω and Q are independent sets of variables. This assumption means that formula 5 can be factorized as

$$G^{(4)}(t_1, t_2, t_3, t_4) = \langle A^2(\Omega_1)E^2(\Omega_2)A^2(\Omega_3)E^2(\Omega_4)\rangle \langle F_a(Q_1)F_e(Q_2)F_a(Q_3)F_e(Q_4)\rangle \quad (6)$$

(A similar assumption was used by Wegener and Rigler [1984] in the theory of fluorescence photobleaching recovery.) Let us call the first factor in this formula the rotational factor, and the second factor would be the isotropic correlation function.

As we have already noted, in fluorescence correlation experiments the second-order correlation function under constant excitation is usually measured. This is expressed as an integral from the fourth-order correlation function:

$$G^{(2)}(\tau) = \int_{-\infty}^{\tau} dt'' \int_{-\infty}^{0} G^{(4)}(t', 0, t'', \tau)dt' = \quad (7)$$

$$= \int_{-\infty}^{\tau} dt'' \int_{-\infty}^{0} \langle A^2(t')E^2(0)A^2(t'')E^2(\tau)\rangle \cdot \langle F_a(t')F_e(0)F_a(t'')F_e(\tau)\rangle\, dt'. \quad (8)$$

To give an idea how the information about the rotational motion of the fluorophore molecule can be obtained from the experiment, we shall make some further assumptions now. We shall assume that the molecule has only a single fluorescence unit. We shall also assume monoexponential decay of fluorescence. Under these assumptions

$$\langle F_a(Q_1)F_e(Q_2)F_a(Q_3)F_e(Q_4)\rangle = S^{(2)}(t_3 - t_1)\tau_e^{-2}\exp[-(t_2 - t_1 + t_4 - t_3)/\tau_e], \text{ if } t_1 \le t_2 \le t_3 \le t_4; \text{ 0 for the other values of } t_3. \quad (9)$$

Here

$$S^{(2)}(t) = \langle F_a(0)F_a(t)\rangle/[1-\exp(-t/\tau_e)] \quad (10)$$

is a slowly decreasing function if compared to the fluorescence decay. (For photostable immobile molecules $S^{(2)}(t)$ = constant. In case of rhodamines the fastest process contributing to the decrease of $S^{(2)}(t)$ is usually the populating of the triplet excited states, which has a relaxation time in the range of microseconds [Kändler et al., 1982].) Therefore, $S^{(2)}(t_3 - t_1)$ in Eq. 9 can well be taken equal to $S^{(2)}(\tau)$. Under this approximation Eq. 8 leads to

$$G^{(2)}(\tau) = S^{(2)}(\tau)\tau_e^{-2}\int_{-\infty}^{0} dt' \int_{0}^{\tau} dt'' \langle A^2(t')E^2(0)A^2(t'')E^2(\tau)\rangle \times \exp[(t' - \tau + t'')/\tau_e]. \quad (11)$$

In Eq. 11, $S^{(2)}(\tau)$ has the meaning of the isotropic correlation function of the classical fluorescence intensity. Photon antibunching is described along with rotational diffusion by the remaining part of Eq. 11. For example, in the case of spherical molecule, this part has been expressed by Aragon and Pecora (1975). According to their expressions,

$$G^{(2)}(\tau) = S^{(2)}(\tau)\sum_{lm}[c_{lm} - d_{lm}\exp(-\tau/\tau_e)] \cdot \exp[-l(l+1)D\tau], \quad (12)$$

where D is the rotational diffusion coefficient and

$$c_{lm} = (4\pi)^{-1}\sum_{\substack{l_1 m_1 \\ l_2 m_2}} \iiiint A^2(\Omega_{t'})E^2(\Omega_0)A^2(\Omega_{t''})E^2(\Omega_\tau) \times \frac{Y_{l_1 m_1}(\Omega_\tau)Y^*_{l_1 m_1}(\Omega_{t''})Y_{lm}(\Omega_{t''})Y^*_{lm}(\Omega_0)Y_{l_2 m_2}(\Omega_0)Y^*_{l_2 m_2}(\Omega_{t'})}{[1 + (l_1(l_1+1) - l(l+1))D\tau_e][1 + l_2(l_2+1)D\tau_e]} \times d\Omega_{t'}\, d\Omega_0\, d\Omega_{t''}\, d\Omega_\tau; \quad (13)$$

$$d_{lm} = (4\pi)^{-1} \sum_{\substack{l_1 m_1 \\ l_2 m_2}} \iiint\int A^2(\Omega_{t'}) E^2(\Omega_0) A^2(\Omega_{t''}) E^2(\Omega_\tau) \times \frac{Y_{lm}(\Omega_\tau) Y^*_{lm}(\Omega_{t''}) Y_{l_1 m_1}(\Omega_{t''}) Y^*_{l_1 m_1}(\Omega_0) Y_{l_2 m_2}(\Omega_0) Y^*_{l_2 m_2}(\Omega_{t'})}{[1 + (l(l+1) - l_1(l_1+1))D\tau_e][1 + l_2(l_2+1)D\tau_e]} \times d\Omega_{t'}\, d\Omega_0\, d\Omega_{t''}\, d\Omega_\tau. \quad (14)$$

Expressions 13 and 14 are rather clumsy. Great simplification of formula 11 can be achieved in the limit

$$\tau_e \to 0. \quad (15)$$

This approach has extensively been used in FCS theory (Ehrenberg and Rigler, 1974; Aragon and Pecora, 1975; 1976; etc.) and is acceptable if fluorescence decay is much faster than rotational relaxation. It permits carrying out the integration in formula 11 taking simply $\Omega_{t'} = \Omega_0$ and $\Omega_{t''} = \Omega_\tau$:

$$G^{(2)}(\tau) \simeq S^{(2)}(\tau)[1 - \exp(-\tau/\tau_e)] \cdot \langle A^2(0)E^2(0)A^2(\tau)E^2(\tau)\rangle. \quad (16)$$

The first factor of formula 16 accounts for the classical intensity correlations of fluorescence of the molecule in the fully unpolarized experiment, the second factor describes a quantum effect in FCS, the photon antibunching phenomenon, and the third factor describes rotational correlations. We shall call the product of the first and the second factors the isotropic factor or the isotropic correlation function.

It should be noted here that assumption 15 is not only needed for the simplification of the outlook of some analytical expressions but is also actually necessary for separation of the rotational contribution as a factor.

Eq. 16 shows the way to separate the rotational factor in the results of experiments. If we were able to carry out the experiment (or to find out the combination of the measured functions) under the conditions when the rotational factor is constant, this would mean that we can independently measure the isotropic factor. Merely the independent measurement of the isotropic factor is the key question even though the rotational factor is our specific interest.

A possible way to determine the isotropic correlation function is to average over all possible polarizations (Kask, 1986). This is an inconvenient way because either the experiment of fully unpolarized excitation or the experiments using a sequence of excitation pulses of different polarization should be required. We shall use another approach to the problem.

The properties of the rotational factor

The rotational factor

$$C(\tau) \equiv \langle A^2(0)E_1^2(0)A^2(\tau)E_2^2(\tau)\rangle \quad (17)$$

depends on the geometry of the experiment, i.e., on relative orientations of excitation polarization ($\hat{\mathbf{a}}$), and of the two polarizations, $\hat{\mathbf{e}}_1$ and $\hat{\mathbf{e}}_2$, of fluorescence detection at time moments 0 and τ, respectively. Of course, the rotational factor depends also upon the properties of the molecule under study. The simplest model is the spherical rigid molecule. Assume also that the two transition moments of the molecule are parallel:

$$\hat{\mu}_a = \hat{\mu}_e = \hat{\mu}. \quad (18)$$

The behavior of the molecule in solution is described by the equation of rotational diffusion

$$dP(\hat{\mu}, t)/dt = DL^2 P(\hat{\mu}, t). \quad (19)$$

L is the angular momentum operator.

The special solution of Eq. 19 under initial condition $\hat{\mu}(0) = \hat{\mu}_0$ is

$$P(\hat{\mu}, t|\hat{\mu}_0) = \sum_{l=0}^{\infty} \sum_{m=-l}^{l} Y_{lm}(\hat{\mu}_0) Y^*_{lm}(\hat{\mu}) \exp[-l(l+1)Dt]. \quad (20)$$

Functions Y_{lm} are the spherical harmonics.

Definition of $C(\tau)$, Eq. 11, gives the expression

$$C(\tau) = \iint A^2(\hat{\mu}_0)E_1^2(\hat{\mu}_0)P(\hat{\mu}_0) \cdot P(\hat{\mu}, \tau|\hat{\mu}_0)A^2(\mu)E_2^2(\hat{\mu})\, d^2\hat{\mu}_0\, d^2\hat{\mu}, \quad (21)$$

where

$$P(\hat{\mu}_0) = (4\pi)^{-1}. \quad (22)$$

Incorporating expressions 20 and 22 into Eq. 21, one gets

$$C(\tau) = (4\pi)^{-1} \sum_{l,m} \exp[-l(l+1)Dt] \int A^2(\hat{\mu}_0)E_1^2(\hat{\mu}_0) \cdot Y_{lm}(\hat{\mu}_0)\, d^2\hat{\mu}_0 \times \int A^2(\hat{\mu})E_2^2(\hat{\mu})Y^*_{lm}(\hat{\mu})\, d^2\hat{\mu} \quad (23)$$

$$\equiv \sum_{l,m} b_{lm}(\hat{\mathbf{a}}, \hat{\mathbf{e}}_1) b^*_{lm}(\hat{\mathbf{a}}, \hat{\mathbf{e}}_2) \exp[-l(l+1)Dt] \quad (24)$$

$$\equiv \sum_{l} B_l(\hat{\mathbf{a}}, \hat{\mathbf{e}}_1, \hat{\mathbf{e}}_2) \exp[-l(l+1)Dt]. \quad (25)$$

Here

$$b_{lm}(\hat{\mathbf{a}}, \hat{\mathbf{e}}) \equiv 2^{-1}\pi^{-1/2} \int A^2(\hat{\mu})E^2(\hat{\mu})Y_{lm}(\hat{\mu})\, d^2\hat{\mu}, \quad (26)$$

$$B_l(\hat{\mathbf{a}}, \hat{\mathbf{e}}_1, \hat{\mathbf{e}}_2) \equiv \sum_{m=-l}^{l} b_{lm}(\hat{\mathbf{a}}, \hat{\mathbf{e}}_1) b^*_{lm}(\hat{\mathbf{a}}, \hat{\mathbf{e}}_2). \quad (27)$$

Choosing the usual relations between the Cartesian (x, y, z) and polar coordinates (θ, ϕ) of unity vector,

$$\left.\begin{aligned} z &= \cos\theta, \\ x &= \sin\theta\cos\phi, \\ y &= \sin\theta\sin\phi, \end{aligned}\right\} \quad (28)$$

and using expressions

$$\left.\begin{aligned} d^2\hat{\mu} &= \sin\theta\, d\theta d\phi, \\ Y_{lm}(\theta,\phi) &= (2\pi)^{-1/2}\Theta_{lm}(\theta)\exp(im\phi), \\ \Theta_{lm}(\theta) &= (-1)^m[(2l+1)(l-m)!/2(l+m)!]^{1/2} \\ &\quad\cdot(\sin^m\theta)\times\partial^m P_l(\cos\theta)/(\partial\cos\theta)^m, \\ P(\cos\theta) &= (l!2^l)^{-1}\partial^l[(\cos^2\theta-1)^l]/[\partial(\cos\theta)]^l, \end{aligned}\right\} \quad (29)$$

$$\left.\begin{aligned} A^2(\hat{x}) &= E^2(\hat{x}) = \sin^2\theta\cos^2\phi, \\ A^2(\hat{y}) &= E^2(\hat{y}) = \sin^2\theta\sin^2\phi, \\ A^2(\hat{z}) &= E^2(\hat{z}) = \cos^2\theta, \end{aligned}\right\} \quad (30)$$

we have carried out straightforward calculations for the values of b_{lm} (Table 1). For even m values,

$$b_{l,-m} = b^*_{l,m}. \quad (31)$$

Coefficients B_l for a number of polarizations can simply be calculated from the data of Table 1 (see Table 2).

The data for the xxx and xyy polarizations agree with the results of Aragon and Pecora (1976). Cross-correlation functions (such as those for xxy and zxy polarizations) have not been calculated before.

Proceeding from the data of Table 2, it is possible to find linear combinations of the correlation functions, for which $B_2 = B_4 = 0$. An example of the isotropic correlation function is

$$G_{isotr} \sim 10G_{zxy} + 2G_{xyy} + G_{xxy}. \quad (32)$$

In the experiments described below the isotropic correlation function is calculated according to a slight modification of Eq. 32:

$$G_{isotr} \sim 20G_{zxy} + 4G_{xyy} + G_{xxy} + G_{xyx}. \quad (33)$$

The reason for the modification is the following. G_{isotr}, like any other autocorrelation function, must be symmetric relative to the zero time axes. According to the simple theory presented above, all the correlation functions are symmetric and the pairs like G_{xxy} and G_{xyx} are equal. Actually, G_{xxy} and G_{xyx} are both asymmetric. To overcome the symmetry problem, we have replaced G_{xxy} of Eq. 24 by a linear combination, $\frac{1}{2}(G_{xxy} + G_{xyx})$, which is definitely symmetric, because $G_{xxy}(\tau) \equiv G_{xyx}(-\tau)$.

TABLE 2 The values of 35^2B_l dependent on the polarizations $\hat{a}\hat{e}_1\hat{e}_2$

	$\hat{a}\hat{e}_1\hat{e}_2$			
l	xxx	xxy	xyy	zxy
0	3,969	1,323	441	441
2	6,480	540	180	−90
4	576	−280	324	−36

The reader is, perhaps, interested why G_{xxy} and G_{xyx} are asymmetric. The source of the asymmetry can be found in the rotational factor of the fourth order correlation function, $\langle A^2(t_1)E^2_1(t_2)A^2(t_3)E^2_2(t_4)\rangle$. Asymmetry of the factor can be pictured by imaging the most favorable rotational movement of a molecule needed for the greatest contribution to correlations. For the xxy polarization, we expect that the transition moment of the molecule was oriented towards, x, x, x, and y axes at the four consecutive time moments when photons are absorbed or emitted, respectively. For the xyx polarization the respective consecutive orientations are x, y, x, and x. In the first case the orientation has to change only once, while in the second case it has to do it twice. Consequently, $G^{(4)}_{xxy}$ $G^{(4)}_{xyx}$ at $t_1 < t_2 < t_3 < t_4$, and $G^{(2)}_{xxy} > G^{(2)}_{xyx}(\tau)$ at $\tau > 0$.

METHODS

Optics and electronics

The apparatus for the experiment (Fig. 1) is nothing but a microfluorimeter equipped with the two photon detectors and a fast correlator device (Kask et al., 1985, 1987). Fluorescence is excited by a focused beam of a continuous wave dye laser at the 575-nm wavelength. The dominant (TEM_{00}) mode of laser action and beam expansion over the aperture of the focusing objective are needed to get a minimal beam radius in focus. Using objective 6.3 × 0.16, a laser beam radius of about 1.5 μm has been achieved.

The sample solution enters the filled cuvette through a syringe needle which is accurately aimed at the focus of observation (Kask et al., 1987). The solution flow, perpendicular to the exciting laser beam, is created in the sample domain by connecting the input and the output of the filled cuvette to two vessels at different solution levels. The flow exchanges the

TABLE 1 The values of $35b_{lm}$ dependent on the polarizations $\hat{a}\hat{e}$

	$\hat{a}\hat{e}$					
lm	xx	yy	zz	xz	xy	yz
00	63	63	63	21	21	21
20	$-18\times5^{1/2}$	$-18\times5^{1/2}$	$36\times5^{1/2}$	$3\times5^{1/2}$	$-6\times5^{1/2}$	$3\times5^{1/2}$
22	$9\times30^{1/2}$	$9\times30^{1/2}$	0	$-3\times30^{1/2}/2$	0	$-3\times30^{1/2}/2$
40	9	9	24	−12	3	−12
42	$3\times10^{1/2}$	$-3\times10^{1/2}$	0	$3\times10^{1/2}$	0	$-3\times10^{1/2}$
44	$3\times70^{1/2}/2$	$3\times70^{1/2}/2$	0	0	$-3\times70^{1/2}/2$	0

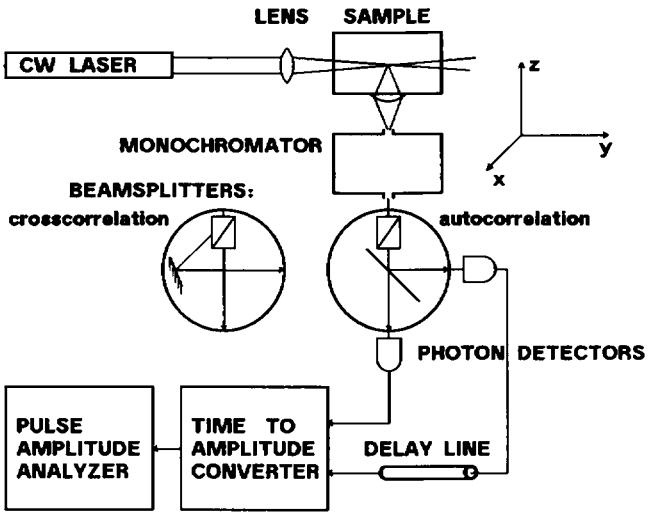

FIGURE 1 Schematic diagram of the FCS apparatus.

bleached molecules for fresh ones. A flow velocity of about 50 mm/s has been used.

Fluorescence is collected at right angles in respect to the exciting light beam with the help of a water-immersion objective 30 × 0.90 (the sample is at a 1.2-mm distance from the objective). A diaphragm is positioned in the image plane of the observation objective to separate a short segment of the image of the fluorescent trace of the laser beam for further use. The sample volume is about 150 μm^3. Emission is optically filtered using a simple prism monochromator which allows removing the scattered light at the laser wavelength, and also the two most intense lines of the Raman scattering from water. The remaining background emission from pure water (approximately 10^{12} water molecules per sample volume) is about equivalent to fluorescence of a single molecule of Texas Red.

The light emerging from the output slit of the monochromator is divided into two beams of certain polarization. If autocorrelation functions (polarizations *xxx* or *xyy*) are measured, a polarizer and a half-transparent beamsplitter are used; if cross-correlation functions (*xxy* or *zxy*) are measured, a birefringence polarizer is used.

The electronic part of the apparatus (Kask et al., 1985) consists of two photon detectors, a time-to-amplitude converter (type 1701, Polon, Nuclear Equipment Establishment, Warsaw, Poland), and a multichannel pulse-amplitude analyzer (type NTA 1024, EMG, Works for Electronic Measuring Gear, Budapest, Hungary). The photon detectors consist of a photomultiplier (type R928, Hamamatsu TV Co., Ltd., Hamamatru, Japan) whose output is fed to a constant fraction discriminator via a preamplifier. The time response function of the system has been measured using short (0.1-ns) pulses from the mode-locked cavity dumped argon ion laser; it has a full width at half maximum of 0.7 ns.

Data reduction (curve fitting including deconvolution) has been carried out on the Cadmus 9200 (Periphere Computer Systeme GmbH, München, FRG) computer.

Sample preparation and characterization

Porcine pancreatic lipase (PL) prepared according to Sikk et al. (1985) was a gift from Dr. P. Sikk. Texas Red (TR) was purchased from Sigma Chemical Co., St. Louis, MO, Tris from Serva Fine Biochemicals, Heidelberg, FRG, and Sephadex G-15 and Sephadex G-100 from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The other reagents were from Reakhim (USSR). All the solutions were made in bidistilled water.

For purposes of labeling, some grains of TR (~0.4–0.5 mg) were added to the solution of 1 mg of PL in 0.9 ml of 0.15 M sodium carbonate buffer, pH 9.0, at 0°C. The mixture was left overnight while being stirred in the dark at 0°C. The excess of the dye was removed on a Sephadex G-15 column, equilibrated with 10 mM sodium acetate, pH 5.8. The main fraction containing labeled protein was rechromatographed on a Sephadex G-100 column equilibrated with 25 mM Tris-HCl, pH 7.0, and colored fractions of 2 ml were collected. All these buffers contained 2 mM $CaCl_2$. The dye-to-protein ratio was estimated by light absorption measurements to be near unity. The solutions for the fluorescence correlation experiments were filtered through a glass filter (1.7 μm, Saale Glas, German Democratic Republic), and contained about 7×10^{-11} M of PL-TR in 25 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.0.

For independent characterization of the rotational diffusion of the prepared material, a common fluorescence method, the time-resolved fluorescence depolarization (TRFD; Tao, 1969) was at first applied at 22°C. Fluorescence intensity decay has a slight deviation from a single exponential law and an average τ_e value of 3.72 ± 0.03 ns. The fluorescence anisotropy decay has at least two exponential terms. The estimated amplitude and the decay time values are $a_1 = 0.053$, $\tau_1 = 0.87$ ± 0.09 ns for the faster term and $a_2 = 0.287$, $\tau_2 = 33.2 \pm 2.0$ ns for the slower term, respectively. According to usual interpretation the slower term characterizes the rotational diffusion of the whole molecule whereas the faster term results from the partial rotational freedom of the label.

The fluorescence polarization ratio $I_\parallel/I_\perp$ was measured to be 2.09.

Experimental procedures

For each fluorescence correlation experiment, 250 ml of solution was prepared, or the amount that flows through our cuvette in 10 h at a flow velocity of 50 mm/s. Laser beam power of 20 mW was employed. The mean photon counting rate varied from 50,000 to 200,000 s^{-1}, depending on the type of the beam divider and the detected light polarization. (Less than a fifth of this was on account of pure solvent.) The apparent mean number of the labeled molecules per sample volume (estimated from the results of the experiments) varied from 5 to 7. The exact number depends on the light polarization (owing to the change of the relative amount of the scattered light), and on some uncontrolled random qualities such as the exact concentration of the labeled protein, the quality of optical adjustment, etc. Integration time of a single experiment was about 10 h. Different polarizations ($\hat{\mathbf{a}}\hat{\mathbf{e}}_1\hat{\mathbf{e}}_2$) were used: *xxx*, *xyy*, *xxy*, *xyx*, and *zxy*. Experiments were run at the 22°C temperature.

Each fluorescence correlation experiment was followed by a long subsequent reference experiment in which light from the incandescent lamp was measured by the same equipment. The result of the reference experiment was used to correct the results of the fluorescence correlation experiments for elimination of instrumental distortions (Kask et al., 1987), caused, for example, by inolinearities of the time-to-amplitude converter and by the dead time effects.

Data reduction

After the correction by the reference experiment, the result is the estimated fluorescence intensity correlation function over a segment of its arguments around the zero value. (The zero argument is positioned at the center of the scale by choosing a delay cable of suitable length in the stop channel; see Fig. 1.)

In the results of the experiments, in addition to the correlation function of single molecule, a constant background is always present. This background level is formed by the scattered light of constant intensity and by the cross-correlation terms of intensities of independent light sources (different molecules). This background level needs to be subtracted. To estimate the value of the background, a criterion, according to which the fluorescence intensity correlation function of a single molecule should be zero at zero argument, was used. Preliminary curve fitting including deconvolution was used to estimate the true value of the baseline.

When the background level had been subtracted, the rest of the correlation function was normalized to get its amplitude into accord with the measured polarization ratio. As the next step, the isotropic correlation function was calculated according to Eq. 33. To calculate the rotational factor for each polarization, the correlation functions of the polarized experiments were divided by the fit curve of the isotropic correlation function.

RESULTS

After background subtraction and normalization, the correlation functions of the polarized experiments look like those in Fig. 2. The isotropic factor calculated according to Eq. 33, can well be fitted by the antibunching factor (see the second factor of Eq. 16) multiplied by a slowly decaying function (Fig. 3). The time constant of the photon antibunching factor of the fit curve is $\tau_a = 3.21 \pm 0.09$ ns. The calculated rotational factors are presented in Fig. 4.

C_{xxx} (Fig. 4) was fitted by the sum of a baseline and two exponential functions. The estimated value of the baseline was $a_0 = 2.41$; the amplitude and the decay time values of the exponential functions were $a_1 = 1.26 \pm 0.04$, $\tau_1 = 5.9 \pm 0.5$ ns, $a_2 = 2.51 \pm 0.06$, $\tau_2 = 33.8 \pm 0.7$ ns. The statistical error values were determined according to the following procedure: (*a*) the result of each cycle of the experiment (G_{xxx}, G_{xyy}, G_{zxy}, and $G_{xxy} + G_{xyx}$) was fitted by a sum of a small number of exponential functions; (*b*) a set of simulated data was generated which differed from the initial fit curves by a stochastic noise similar to the noise of experiments; (*c*) the same data reduction procedure as that used for the data of original experiments was applied to the set of the simulated data; (*d*) data simulation and fit procedures were repeated several times; (*e*) the root mean value of the deviations was calculated and assigned to the statistical error value of the respective fit parameter.

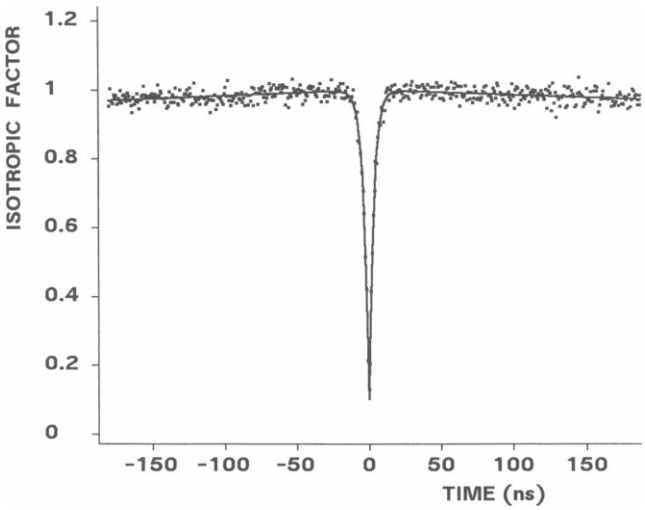

FIGURE 3 The isotropic factor calculated according to Eq. (33).

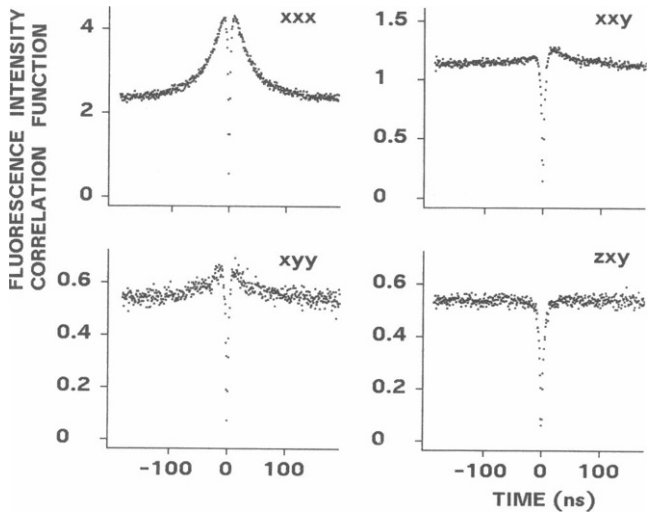

FIGURE 2 The measured fluorescence intensity correlation functions of the PL-TR molecule.

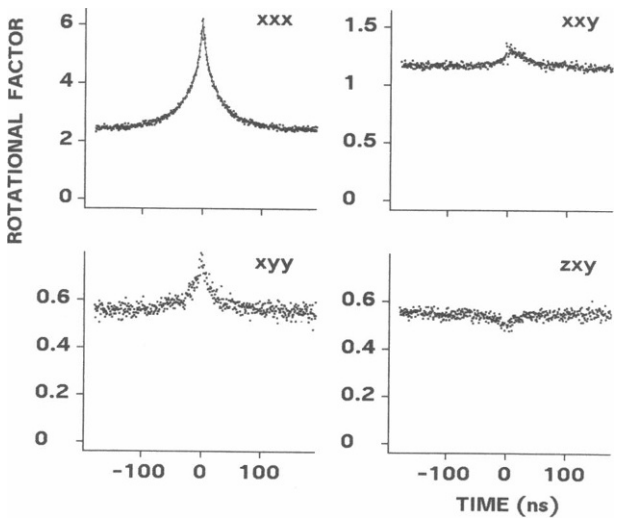

FIGURE 4 The rotational factors of the PL-TR molecule (experimental data).

DISCUSSION

The rather simple theory of separation of rotational and isotropic factors, presented above (Eq. 16), assumes that the fluorescence lifetime is much shorter than the rotational correlation time of the molecule. Therefore the labeled lipase in water is not a proper model object for the respective illustrative experiments because the label has some segmental rotational flexibility (corresponding to the $\tau_1 = 0.9$ ns term of the TRFD experiment), which is faster than fluorescence decay. Nevertheless, we carried out the experiments because, for the studies in the time range where rotational relaxation of labeled proteins occurs, we can use the other fluorescence method, TRFD, for independent characterization of the sample.

To understand what kind of complications are expected if the simple theory is applied instead of the more general one, one has to compare Eq. 16 with Eq. 12. In Eq. 16 the fluorescence antibunching and the rotational diffusion contributions are separated as factors whereas in Eq. 12 their interference is more complex. According to the more general formula 12, these contributions factor only at $|\tau| \gg \tau_c$. Hence, if the assumptions of Eq. 12 are not met, then the calculation of the rotational contribution by dividing the measured correlation function by the isotropic correlation function is justified only at $|\tau| \gg \tau_c$.

Therefore the short-time edge of the C_{xxx} (described by the term $\tau_1 = 5.9$ ns) should not be taken too seriously. (Actually one should expect two noticeable terms at the short-time edge of the rotational factor: a small $l = 4$ term having the decay time value of about 10 ns, and a term corresponding to the faster term of the TRFD experiment having the decay time value of about 0.9 ns.)

The decay time value of the slower term of C_{xxx}, $\tau_2 =$ 33.8 ns, coincides with the respective data of the TRFD experiment, $\tau_2 = 33.2$ ns.

Although Eq. 33 has also been derived under assumption 15, the isotropic factor calculated according to Eq. 33 looks correct. At least evident contribution from slow rotational motion is absent. This is clear when one compares the correlation function of Fig. 3 with that of Fig. 2 (*upper left*): the latter has a strong rotational term decaying with the time constant of about 34 ns which is practically absent in the Fig. 3. The time constant of the photon antibunching factor, $\tau_a = 3.2$ ns, is a bit shorter than fluorescence lifetime, $\tau_c = 3.7$ ns. The reason is the high excitation intensity: τ_a is equal to $(\tau_c^{-1} + I\sigma)^{-1}$ (I—light intensity, σ—absorption cross section of the molecule; Kask, 1987) rather than to τ_c of Eq. 9 (which assumes low I value). We believe that the very slow decay of the isotropic factor is the effect of reversible photobleaching (Kändler et al., 1982); it has a correlation time value of about 1 μs.

The simple theory of rotational factor presented above cannot be directly used for curve fitting: for example, the theory rigidly predicts the value of the polarization ratio of 3.0. (This value corresponds to $\tau_c \rightarrow 0$. To obtain more realistic amplitude values of different terms of the correlation functions, in case of rigid spherical rotators cumbersome Eqs. 13 and 14 should be used. The rotational behavior of the labeled protein molecules is even more complex.) Nevertheless, the theoretical rotational factors calculated from the data of the Table 2 are also presented (Fig. 5). The $(6D)^{-1}$ value of 33.8 ns was chosen in the calculations in accordance with the decay time of the slower term of the measured C_{xxx}. The similarity between Fig. 4 and Fig. 5 is evident.

It should be noted that the criterion $G(0) = 0$ is not the only possible one for separating the contribution of a single molecule in the intensity correlation function. In longer time ranges, the criterion $G(\infty) = 0$ is a more practical one and can be used instead.

A second point is about the long integration times needed in the experiments reported above. Integration times extending to hours are often impractical. However, according to signal-to-noise calculations (Koppel, 1974), the situation is rather different for the studies in the intermediate time ranges, from 10^{-7} to 10^{-2} s, where a few minutes of integration time should usually be sufficient.

The results presented in Fig. 2 prove that Texas Red is a label for which the fluorescence intensity correlation functions at different polarizations can be measured with sufficient precision even in the nanosecond time range.

As it has been shown, the rotational contribution can well be separated from the other contributions such as those of photobleaching, chemical transformations, or

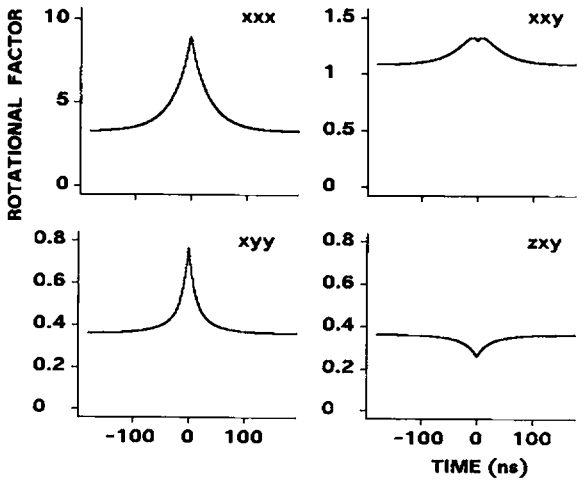

FIGURE 5 The rotational factors of a rigid molecule (theoretical calculations).

translational motion. Applications of FCS to rotational motion are possible in a very broad range of correlation times, from 10^{-8} to 1 s, which differs substantially from the range of applications of TRFD.

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