FLUORESCENCE ENERGY TRANSFER FROM DIPHENYLHEXATRIENE TO BACTERIORHODOPSIN IN LIPID VESICLES

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ABSTRACT Fluorescence energy transfer between the donor diphenylhexatriene (DPH) and the acceptor retinal and fluorescence depolarization of DPH are used to test current theories for fluorescence energy transfer in two-dimensional systems and to obtain information on the effect of the intrinsic membrane protein, bacteriorhodopsin, on the order and dynamics of the lipid phase. Increasing the surface concentration of acceptors by raising the protein to lipid ratio leads to a decrease in the mean fluorescence lifetime by up to a factor of four. When the acceptor concentration is reduced at a fixed protein to lipid ratio by photochemical destruction of retinal, the lifetime increases and reaches approximately the value observed in protein-free vesicles when the bleaching is complete. The shape of the decay curve and the dependency of the mean lifetime on the surface concentration of acceptors are in agreement with theoretical predictions for a two-dimensional random distribution of donors and acceptors. From this analysis a distance of closest approach between donors and acceptors of ~18 Å is obtained, which is close to the effective radius of bacteriorhodopsin (17 Å) and consistent with current ideas about the location of retinal in the interior of the protein. In the absence of energy transfer (bleached vesicles), the steady-state fluorescence anisotropy, $\bar{r}$, of DPH is considerably lower than in the corresponding unbleached vesicles, indicating that the effect of energy transfer must be taken into account when interpreting $\bar{r}$ in terms of order and dynamics.

INTRODUCTION

Fluorescence energy transfer between the lipid and protein components of a membrane allows us to obtain interesting information regarding acceptor surface concentration, lipid lateral phase separation, lipid-protein interactions, and distances of closest approach. We have tested current theories for energy transfer in two dimensions (1, 2, 3, 4, 5) using a vesicle system consisting of the synthetic lipid dimyristoylphosphatidylcholine (DMPC) and the intrinsic membrane protein, bacteriorhodopsin (BR). Large unilamellar vesicles (0.25-μm radius) of variable lipid to protein ratios were prepared (6, 7, 8). On the basis of circular dichroism and measurements of the rotational diffusion of BR, it was shown that BR is monomeric above the phase transition temperature (T_c) of the lipids (6, 7). We can thus assume that BR is randomly distributed in the plane of the bilayer. ~6 ° below T_c, a BR lattice is formed that has the same unit-cell dimensions as in the purple membrane (6, 9). BR is functional as a light-driven proton pump in these vesicles (10). Using nanosecond fluorescence lifetime measurements, we established that fluorescence energy transfer occurs between the donor 1,6-diphenyl-1,3,5-hexatriene (DPH) in the lipid phase and the acceptor retinal of BR (one per BR) (see also reference 32). Above T_c the dependence of the quantum yield of the donor on the surface concentration of the acceptor and the time dependence of the decay of the donor are in good agreement with predictions for a random two-dimensional distribution of donors and acceptors. The interpretation of energy-transfer experiments is often complicated by uncertainties regarding the orientation factor (11). In the present case the rapid and extensive wobbling of the donor during the fluorescence lifetime ensures that the orientation factor is between 0.44 and 0.56 and never approaches 0 above T_c. If retinal is buried within BR, the extent of energy transfer will be less than in the case where retinal is on the surface of BR and donor and acceptor can get very close together. This effect was employed to determine the distance of closest approach between DPH and retinal within the framework of a model in which BR is approximated by a cylinder with retinal at the center.

Both structural and dynamical information on the lipid phase can be obtained from time-dependent and steady-state fluorescence depolarization experiments with lipid soluble probes like DPH (7, 12, 13). Energy transfer may

have a significant effect in such measurements, because the decrease in the lifetime of the donor reduces the time over which rotational diffusion can lead to depolarization, thus raising the fluorescence anisotropy. We show experimentally that this effect indeed occurs and cannot be neglected.

MATERIALS AND METHODS

DMPC vesicles containing BR at various BR/DMPC ratios were prepared according to a procedure described elsewhere (6, 8). All measurements were made in 50-mM phosphate buffer, pH 6.9. The fluorescence background was ~1%. The chromophore retinal was destroyed by bleaching with a 300-W xenon lamp (VIX-300UV; Varian Associates, Palo Alto, CA) using two heat filters (KG 2; Schott Optical Glass Inc., Mainz, Federal Republic of Germany) transmitting between 320 and 770 nm (¼ W/cm² at the sample). In this wavelength range no spectral contribution of retinal or its products was observable after 2 h of illumination. The vesicle solution with the highest BR/DMPC ratio was divided into four samples that were partially bleached to different extents. The chromophore content remaining in these samples after bleaching, as monitored by the absorbance at 570 nm, was 63, 30, 181, and 0% of the original amount.

DPH was incorporated into the vesicles by incubation for 30 min at 30°C in a 500:1 DMPC/DPH ratio immediately before the measurements. Steady-state fluorescence depolarization measurements were performed as described previously (7). Fluorescence lifetime measurements were carried out on a single-photon counting instrument with a photomultiplier (XP 2020 Q; Philips Electronic Instruments, Inc., Mahwah, NJ) and Ortec electronics (Ortec, Oak Ridge, TN). The light source was a nanosecond-gated flashlamp (Applied Photophysics Ltd., London, England) modified to reduce the pulsewidth to 1.6 ns full width at half maximum (FWHM). The lamp was run at 4.5 kV, 30 kHz with H_2 gas at a pressure of 0.75 bar, and a spark gap of 0.8 mm. The excitation wavelength was selected with a narrow bandpass filter (362 nm; Balzers-Union, Inc., Balzers, Liechtenstein), the emission wavelength with two low fluorescent cutoff filters (KV 418; Schott Optical Glass Inc.). Data analysis was performed on a PDP 11/40 computer (Digital Equipment Corp., Maynard, MA) interfaced to the multichannel analyzer, using a Marquardt nonlinear least squares algorithm with χ^2_ν to test the goodness of fit (14, 15).

RESULTS AND DISCUSSION

Fluorescence energy transfer is characterized by the parameter R_0, which is the distance between donor and acceptor at which the transfer efficiency is 50% (16). R_0 depends on the quantum yield of the donor in the absence of the acceptor, the index of refraction, n, the spectral overlap integral, J, and the orientation factor, K^2 (16). From the emission spectrum of DPH and the absorption spectrum of BR (dark-adapted) we calculate by numerical integration a value of 7.3×10^{-14} cm³ M^{-1} for J. The orientation factor, K^2, depends on the degree of rotational freedom of the donor and acceptor during the excited-state lifetime. We will show below that for our vesicle preparations K^2 varies between 0.44 and 0.56 depending on the protein content. The extent of wobbling of the donor depends on the lipid to protein ratio and can be estimated from measurements of the fluorescence depolarization of the donor. For the vesicles with the highest protein content we find in this way $K^2 = 0.44$. This point will be discussed in more detail below. Using this value for K^2 together with $n = 1.44$ and a quantum yield of 1, we find that R_0 equals 43 Å. Because the equivalent radius of BR is only ~17 Å, no matter where retinal is located within BR, the range of energy transfer extends well into the lipid phase.

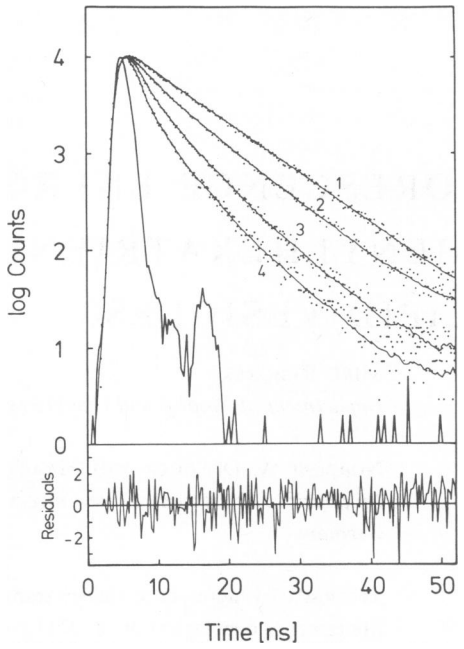

FIGURE 1 Lamp pulse and fluorescence decays (···) of DPH in DMPC vesicles as a function of the molar BR/DMPC ratio at 35°C. The molar ratios are (*top to bottom*) 0 (vesicles of sample 1), (1:331) ± 8.2% (vesicles of sample 2), (1:171) ± 9.2% (vesicles of sample 3), and (1:102) ± 6.3% (vesicles of sample 4). The data points were fitted with a sum of exponentials convoluted with the excitation profile (——). *Bottom*, the weighted residuals for vesicles of sample 4.

Fig. 1 shows the fluorescence decay of DPH at 35°C in four vesicle preparations of varying BR/DMPC ratios. At this temperature BR is monomeric (6, 7). The vesicles are numbered with increasing molar BR to DMPC ratios from 1 to 4 (Table I). For vesicles of sample 1 without protein, the decay is single exponential with a lifetime of 8.24 ns. When the BR/DMPC ratio is increased the decay becomes faster and deviates increasingly from a single exponential. The fluorescence is progressively quenched by the addition of BR. The decays were fitted by a sum of

TABLE I
MOLAR BACTERIORHODOPSIN TO LIPID RATIOS (BR/DMPC), MEAN LIFETIMES, AND χ^2_ν VALUES

Vesicle sample number	BR/DMPC	$\bar{\tau}$	$\bar{\tau}_{bleached}$	χ^2_ν
		ns	*ns*	
1	0	8.24 ± 0.03	—	1.24
2	1/331	5.29 ± 0.34	8.69 ± 0.59	1.14
3	1/171	2.43 ± 0.27	8.01 ± 0.73	1.05
4	1/102	2.01 ± 0.16	7.39 ± 0.25	1.24

Data taken at 35°C.

exponentials ($\Sigma_i \alpha_i \exp - t/\tau_i$). The amplitudes, α_i, and relaxation times, τ_i, have no physical significance in terms of species or processes and are just curve-fitting parameters. Up to a maximum of three exponentials were required for a good fit. To give an indication of the quality of the fit we plotted the residuals for vesicles of sample 4 in Fig. 1. The χ^2 values for the four decays were all close to 1 (Table I). The data of Fig. 1 can be explained on the basis of energy transfer from DPH to the retinal chromophore of BR, because with increasing BR/DMPC ratio the surface concentration of acceptors and thus the number of acceptors within R_0 radius of the donors increases. Further experimental evidence in favor of energy transfer was obtained from experiments with the same vesicles after complete destruction of the acceptor by bleaching. The decay of DPH in these bleached vesicles is again single exponential with a lifetime close to that for the pure lipids, as expected for energy transfer when the acceptor is removed (decay data not shown; mean lifetimes in Fig. 2 *a* and Table I). These experiments also indicate that the BR-induced fluorescence quenching is not due to binding of DPH to the protein.

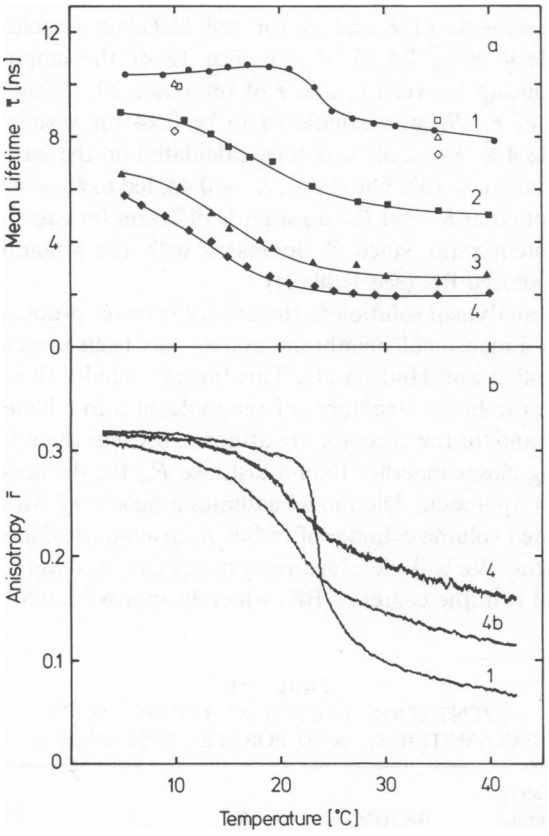

FIGURE 2 (*a*) Temperature dependence of the mean lifetimes of DPH in vesicles of increasing BR/DMPC ratios: vesicles 1 (●), 2 (■), 3 (▲), 4 (◆) (see legend of Fig. 1), and for the corresponding samples where the acceptor-chromophore retinal has been removed by bleaching (△, ◇, and □; data at 5 and 35°C). (*b*) Comparison of the effect of BR and bleached BR on the phase-transition curve of DMPC as monitored by the steady-state fluorescence anisotropy, $\bar{r}$, of DPH. Curve 1, vesicles of sample 1 (pure lipids); curve 4, vesicles of sample 4; curve 4b, vesicles of sample 4 bleached.

Similar experiments were performed at a large number of temperatures between 5 and 40°C. The mean lifetimes, $\bar{\tau}$, for these decays are plotted in Fig. 2 *a* as a function of temperature. The mean lifetime ($\bar{\tau} = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i$) is equal to the area under the normalized decay curve and thus represents the relative quantum yield. The ratio of any two $\bar{\tau}$'s thus equals the ratio of the corresponding quantum yields. From Fig. 2 *a* we note that at each temperature the mean lifetime decreases with increasing BR/DMPC ratio. For vesicles without BR, $\bar{\tau}$ drops near the lipid-phase transition (23.5°C) followed by a further gradual decrease at higher temperature. In the presence of BR, on the other hand, the major decrease in $\bar{\tau}$ occurs at lower temperatures and seems to be associated with the disaggregation of the BR lattice in monomers (17°C) rather than with the lipid-phase transition (7). The open symbols in Fig. 2 *a* are the mean lifetimes for the control experiments with completely bleached vesicles. Except for one point, all of them are very close to the lifetimes for the pure lipids at the same temperature, as expected when the acceptors for energy transfer are removed.

Fig. 2 *b* illustrates the effect that the removal of the acceptor retinal has on the steady-state anisotropy, $\bar{r}$, of the donor DPH. At all temperatures the anisotropy for vesicles with acceptor (vesicles of sample 4) is considerably higher than for the same vesicles after complete bleaching (vesicles of sample 4, bleached). The effect is largest at high temperatures where energy transfer is most effective. It is intuitively clear and may also be seen from Eq. 6 of reference 12, that energy transfer through its reduction in the lifetime leads to an increase in $\bar{r}$. Fig. 2 *b* clearly shows that if the steady-state anisotropy is used to calculate the lipid order parameter and correlation time, the data have to be corrected for the effect of energy transfer. The same holds true for other membrane systems with natural acceptors (cytochromes, rhodopsin). Because no corrections are required with bleached vesicles, the effect of BR on the structural and dynamical properties of the lipids can be studied best with such vesicles, provided that removal of the acceptor does not change the lipid-protein interactions. The complete set of data for all the bleached vesicles (data not shown) confirm our previous observation that with increasing protein to lipid ratio, $\bar{r}$ increases above T_c (7, 12). As shown in Fig. 2 *b*, the effect is smaller than with the corresponding unbleached vesicles. Assuming that the properties of the lipids are homogeneous throughout the bilayer and that removal of the acceptor does not change the limiting anisotropy r_∞ and the rotational correlation time of DPH, we conclude from a comparison of $\bar{r}$ and $\bar{\tau}$ in the bleached and unbleached vesicles that the effect of the protein is primarily to increase r_∞ and thus the probe order parameter. Below T_c we observe with the bleached vesicles a small but gradual decrease in $\bar{r}$ with increasing protein content.

Fluorescence energy transfer between donors and acceptors, which are randomly distributed in a lipid bilayer, can be described by the theory of Förster energy transfer in two dimensions (1, 2, 3, 4, 5). The major difference between the idealized model system in this theory and a membrane is the finite thickness of the bilayer, which might lead to donors and acceptors being positioned at different depths in the bilayer. Although DPH is located in the hydrophobic part of the bilayer, its position and distribution in the direction perpendicular to the bilayer are unknown. Fluorescence depolarization experiments indicate that DPH is wobbling in a cone of half-angle θ_c, whose value depends on the state of the membrane (18, 19). Cone angles between 58 and 72° have been measured for DPH in DMPC and DPPC bilayers above the phase transition (17, 19, 20, 21). The cone axis was found to be normal to the membrane (22), but its vertex is not necessarily in the middle of the bilayer. From a combination of measurements (sequence [23], neutron diffraction [24, 25], fluorescence [26], linear and transient dichroism [27]) the acceptor retinal is believed to be located at a fixed position approximately in the center of the BR with the transition dipole moment of the 568-nm absorption band making an angle of 71 to 78° with the normal of the membrane.

We will assume that the acceptors are randomly distributed in a plane with their transition dipole moments fixed at an angle θ with respect to the normal of this plane, whereas the donors are wobbling in cones of an angle θ_c (18) and are also randomly distributed in the plane defined by the acceptors. The correlation time, τ_c, for the wobbling motion of DPH in this cone (~1 ns [20, 21]) is much faster than its fluorescence lifetime (~8 ns), so that the transfer efficiency for a donor-acceptor pair is determined by the dynamic average of the orientation factor, K^2, over all allowed configurations in the cone (11). The value of this dynamic average can be calculated and expressed in terms of the spherical-polar coordinates, θ and ϕ, of the acceptor transition dipole moment (the z-axis of the coordinate system coincides with the membrane normal; the vector connecting the donor and the acceptor lies in the plane of the membrane along $\phi = 0°$) and the order parameter, S_D, of the donor DPH (12, 13, 18, 33)

$$\overline{K}^2(\phi) = [\tfrac{1}{2}(3\cos^2\phi + 1)]\sin^2\theta[\tfrac{2}{3}(1 - S_D)] + \cos^2\theta[\tfrac{1}{3}(1 + 2S_D)] \quad (1)$$

$$S_D = \tfrac{1}{2}\cos\theta_c(1 + \cos\theta_c). \quad (2)$$

θ lies between 71 and 78°. Above T_c, S_D is <0.5 (17, 21, 28). As seen from Eq. 1, for the given values of θ and S_D, $\overline{K}^2(\phi)$ does not get close to zero for any value of ϕ. The major problem with the K^2 factor is therefore avoided (16). The lateral diffusion of DPH and BR and the rotational diffusion of BR (rotational relaxation time ~15 μs [7]) are slow compared with the lifetime of DPH, so that the position of each cone in the plane, with respect to the acceptor and the orientation of the acceptor transition dipole moment with respect to the vector connecting the retinal and the cone, does not change during the DPH emission. The observed transfer efficiency is thus the static average over the ensemble of all donor-acceptor pairs (11) (average over ϕ). From the 50% value of the efficiency we calculate an apparent dynamic and static average of K^2 that is between 0.44 and 0.56 depending on the choice of S_D and θ. The most uncertain parameter in the calculation of $\overline{K}^2$ is the exact value of S_D, the order parameter of the donor DPH in the presence of protein. One way to determine S_D directly is to measure r_∞, the limiting anisotropy in time-dependent fluorescence anisotropy measurements (12, 13). It is also possible to get a good estimate of S_D from the steady-state anisotropy measurements with bleached vesicles (no energy transfer, Fig. 2, curve 4 *b*) under the assumption that the incorporation of proteins into lipid vesicles only leads to an increase of r_∞ and has no effect on the rotational correlation time τ_c above T_c. This assumption holds true for the case of cytochrome oxidase (28). With this assumption, we estimated r_∞ from the measurements of $\bar{r}$ and τ_F for the bleached vesicles of sample 4 using Eq. 6 of reference 12 or the empirical relationship between r_∞ and $\bar{r}$ of reference 29. Using this value of r_∞, S_D was calculated to be 0.44 for vesicles of sample 4 at 35°C. $\overline{K}^2$ was then calculated on the basis of Eq. 1 with $\theta = 75°$. The result, $\overline{K}^2 = 0.44$, led to $R_0 = 43$ Å. We note that $\overline{K}^2$ and R_0 are slightly different for each lipid to protein ratio, since S_D increases with the amount of incorporated BR (see Table II).

An analytical solution to the energy transfer problem in a two-dimensional membrane system has been presented by Wolber and Hudson (1). This theory includes the case where the bulky structures of the molecules to which the donor and/or the acceptor are attached prevent them from getting closer together than a distance R_e, the distance of closest approach. The model assumes a geometry with an excluded volume cylinder of radius R_e around the donor or acceptor. We will therefore analyze our data assuming the retinal is in the center of BR, which is approximated by a

TABLE II
ORIENTATION FACTOR $\overline{K}^2$, DONOR ORDER PARAMETER S_D, AND FÖRSTER DISTANCE R_0

Vesicle sample number	BR/DMPC	S_D	$\overline{K}^2$	R_0
				Å
1	0	0.25	0.564	45.1
2	1/331	0.30	0.531	44.6
3	1/171	0.41	0.458	43.6
4	1/102	0.44	0.439	43.2

Data taken at 35°C.

cylinder with the same surface area as was calculated from the electron-density map (31). The radius of this cylinder is ~17 Å. The only parameters that enter the theory are the lifetime of the donor in the absence of acceptor, τ, the surface concentration of acceptor, c, R_o, and R_e. τ, c, and R_o can be obtained from independent measurements. The theory predicts the relative quantum yield as a function of $R_o^2 c$ and $R_e/R_o \cdot R_o^2 c$, the number of acceptors per R_o^2 is the natural dimensionless parameter characterizing the energy transfer. The decay is predicted to be exponential with a complex function of τ, $R_o^2 c$, R_e/R_o, and the third root of the time in the exponent (1). The relative quantum yield in the presence and absence of acceptor was obtained from the ratio of the corresponding mean lifetimes. The surface concentration, c, the number of acceptors divided by the surface area of the vesicles, was calculated from the BR/DMPC ratio using a surface area of 60 Å^2 for DMPC (30), and 875 Å^2 for BR (31). Fig. 3 shows the relative quantum yield at 35°C for vesicles of samples 2, 3, and 4 (relative to vesicles of sample 1, the sample with no BR), and for vesicles of sample 4 where the acceptor concentration was reduced to 63, 30, and 18% by bleaching (open symbols, ◊). The horizontal error bars are calculated from the 95% confidence limits of the determination of the BR/DMPC ratio, and the vertical error bars from the uncertainty in the determination of the parameters in the nonlinear least squares fit to the decay data (15). The solid, the dotted, and the dashed line in Fig. 3 are theoretical curves calculated from Eq. 17 of reference 1 for $R_e = 0$, $R_e = 0.25\ R_o$, and $R_e = 0.5\ R_o$, respectively. Except for one preparation, the data points are clearly above the curve for $R_e = 0$ indicating that the distance of closest approach is not negligible. Owing to the $1/R^6$ dependency of the transfer efficiency, significant deviations from the curve with $R_e = 0$ will only occur when R_e is $> 0.25\ R_o$. Of the six data points in Fig. 3, four belong to vesicles of sample 4 at various stages of bleaching (♦ and ◊). Since in these four measurements only the percentage of bleaching was varied, at fixed BR/DMPC ratio, these data points are preferred for a calculation of R_e over the data from vesicles of samples 2 and 3, which are at a different R_o and BR/DMPC ratios. The result of an interpolation calculation for R_e, using the series of vesicles of sample 4 with decreasing acceptor concentration (♦ and ◊), is (0.43 ± 0.11) R_o. Thus $R_e = 18 \pm 5$ Å. The radius of the cylinder of excluded volume around the acceptor is thus about the same as the effective cylindrical radius of BR (17 Å). We conclude that the data analyzed on the basis of this cylindrical geometry are in accordance with the assumption that retinal is in the center of BR rather than on the surface.

A further critical test of the theory used here and of the value of R_e obtained, consists of a comparison of the experimental and theoretical decay curves (Fig. 4). The fluorescence decay of DPH in the absence of acceptor (vesicles of sample 1, ---) is single exponential whereas in the presence of acceptor the decay is clearly curved (---). When $R_e \neq 0$, the analytical expression for the decay

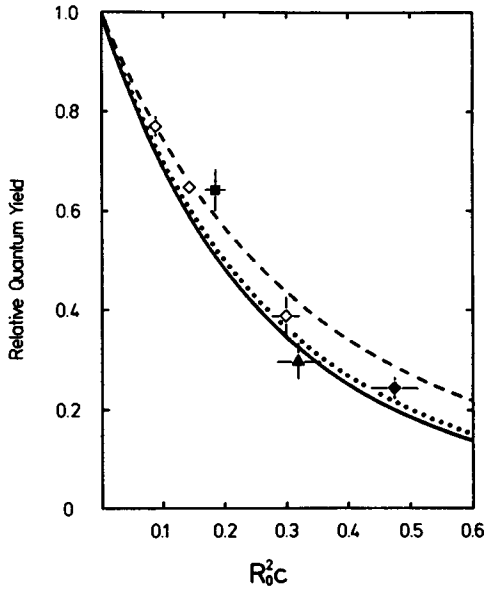

FIGURE 3 Relative quantum yield of DPH (calculated from the ratio of the mean lifetimes in the presence and absence of acceptor) vs. the number of acceptors per R_o^2 ($R_o^2 c$): vesicles of samples 2 (■), 3 (▲), 4 (♦) (see legend of Fig. 1), and vesicles of sample 4 with partially bleached retinal (◊) at 35°C. The solid (——), the dotted (···), and the dashed curve (---) are calculated from Eq. 17 of reference 1 for $R_e = 0$, $R_e = 0.25\ R_o$, and $R_e = 0.5\ R_o$, respectively.

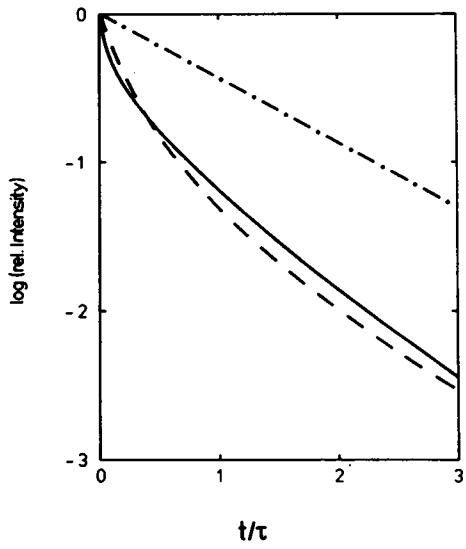

FIGURE 4 Time dependence of the fluorescence decay of DPH at 35°C in the absence (---, vesicles of sample 1) and presence of acceptor (---, vesicles of sample 4) plotted from the corresponding fitting parameters. The time is expressed in units of τ, the fluorescence lifetime in the absence of acceptor (vesicles of sample 4, bleached). Theoretical prediction according to Eq. 21 of reference 1 calculated with $R_e = 0.43\ R_o$ (——). *Rel*, relative.

curves are too complex to use directly in the nonlinear least squares fit. Therefore we deconvoluted the experimental decay curves with a sum of exponentials and compared it with the theory using the same value of R_e = 18 Å as determined from the quantum-yield data. The dashed line of Fig. 4 (---) represents the fluorescence decay of vesicles of sample 4 at 35°C after deconvolution. The time axis is normalized to the lifetime of DPH in the absence of acceptor (vesicles of sample 4, bleached). The theoretical decay curve for $R_e = 0.43\ R_0$ (———) is plotted according to Eq. 21 of reference 1 using the same $R_o^2 c = 0.475$ as was used for vesicles of sample 4 in Fig. 3 (♦). We note that the logarithmic representation of the decay misrepresents the degree of agreement between theory and experiment. The deviation from the theory might be due to the difficulty in measuring very short lived components (<1 ns) with a lamp-pulse width of ~1.6 ns. However the theory provides a reasonable description of the time decay.

Further promising applications of energy transfer may be expected in the following direction: by changing the spectral properties of the donor/acceptor pair and by removal of the acceptor, neighborhoods of varying radius around a protein-bound acceptor will be quenched. In combination with time-resolved anisotropy measurements it may be feasible to learn more about lateral inhomogeneities in the structural and dynamical properties of the lipids surrounding membrane proteins. In conclusion we note that the same approach to determine R_e may be used with other membrane proteins that contain functionally important intrinsic chromophores.

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