Femtosecond Fluorescence Depletion Anisotropy: Application to the B850 Antenna Complex of *Rhodobacter sphaeroides*†

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We extend the technique of fluorescence depletion to measure femtosecond anisotropy decays. In cases where excited-state absorption contributes to the transient spectra, such as in photosynthetic antenna complexes, the anisotropy measured by fluorescence depletion differs from that measured by pump−probe spectroscopy. This new technique has the potential to elucidate the fate of the states created by excited-state absorption.

Introduction

Anisotropy decay on the femtosecond time scale stems from reorientation of transition dipoles by energy transfer, relaxations, or dephasing. The dynamics of these processes typically are studied by measuring the anisotropy of upconverted fluorescence[1] or time-resolved changes in absorption.[2,3] Fluorescence measurements convey information on the transition dipole for spontaneous emission, whereas transient absorption measures combined responses of transition dipoles for ground-state bleaching (GSB), stimulated emission (SE), and excited-state absorption (ESA). In some cases the signals contributing to transient absorption can be resolved spectrally, so that relaxations of the different dipoles can be followed separately. In the case of photosynthetic antenna complexes, however, the ESA spectrum largely overlaps the SE and GSB spectra,[4] making it difficult to untangle ESA anisotropy from the other contributions. Other means of probing anisotropy would therefore be useful.

In this Letter we introduce a new technique for measuring anisotropy decays, illustrate its application to a relatively simple molecule that has no significant ESA in the wavelength range studied, and extend the method to the LH2 antenna complex of the photosynthetic bacterium *Rhodobacter sphaeroides*. We show that the contribution of ESA to the total anisotropy is different than in transient absorption experiments. A comparison of the anisotropy measured by the two methods makes it possible to determine the ESA anisotropy within an assumed model for the photodynamics. The method also provides information on the fate of the higher-energy states generated by ESA.

The concept behind measuring anisotropy by fluorescence depletion is simple. Consider a sample that is excited by two weak, linearly-polarized pulses separated by a time Δt that is short relative to the fluorescence lifetime. For now, we assume that Δt is longer than the pulse widths and the electronic dephasing time, so that we can neglect coherent coupling of the fields of the two pulses. The first pulse selectively excites molecules with certain orientations to an excited singlet state (S_1), generating a time-dependent anisotropy in the sample. The second pulse raises additional molecules to S_1, but also causes some of the molecules in S_1 to decay to the ground state (S_0) by SE and promotes other molecules to higher excited states (S_n) by ESA. Molecules raised to S_n may either relax back to S_1 or decay nonradiatively to S_0 by a variety of pathways, depending on the system. Either SE or ESA thus can decrease the spontaneous fluorescence from molecules that are excited by the first pulse. In addition, depletion of the ground-state population by the first pulse reduces the number of molecules that the second pulse can excite to S_1. The total spontaneous fluorescence resulting from the pair of pulses, integrated over time, thus depends on the relative polarizations of the pulses and on the anisotropy of the sample at the time of the second pulse.

Methods

Pulses peaking at 830 nm, with fwhm ∼30 fs, spectral fwhm ∼850 cm^{-1}, energy 0.7 nJ, and repetition rate 100 kHz, were generated with a Ti:sapphire laser[4] and were split into two pulses (*a* and *b*) of nearly equal intensities. Pulse *a* was polarized horizontally for all the measurements; the polarization of *b* was varied by rotating a zero-order half-wave plate. The two pulses were crossed at ∼5° in the sample, which was held in a 1 cm path length quartz cuvette containing a small magnetic stir bar. A photodiode located above the cuvette detected fluorescence emitted normal to the excitation pulses. To reduce the background fluorescence, the top of the cuvette was masked with black tape except for a 1 mm slit above the intersection of the pulses, which was close to the front wall of the cuvette. (The interaction length of the pulses in the sample was measured previously to be ∼0.15 mm.[4]) The fluorescence was passed through a Polaroid filter to select the detection polarization and a color filter to reject scattered excitation light, and was focused on the photodiode with a 1 cm focal length lens. The two pulse trains were modulated at different frequencies, and the photodiode signal was demodulated at the sum frequency. This procedure automatically subtracts the fluorescence due to the pump and dump pulses acting individually, leaving largely the fluorescence-depletion signal. To measure the total fluorescence without moving the photodiode, measurements must be made with two orientations of the detection polarizer for each polarization of pulse *b* relative to *a*.[5]

The laser dye IR132 (Exciton) was dissolved in dimethyl sulfoxide. Chromatophore membranes containing LH2 antenna complexes were obtained as described[6] from *Rb. sphaeroides* strain ΔQBALM/Q, which lacks reaction centers and LH1

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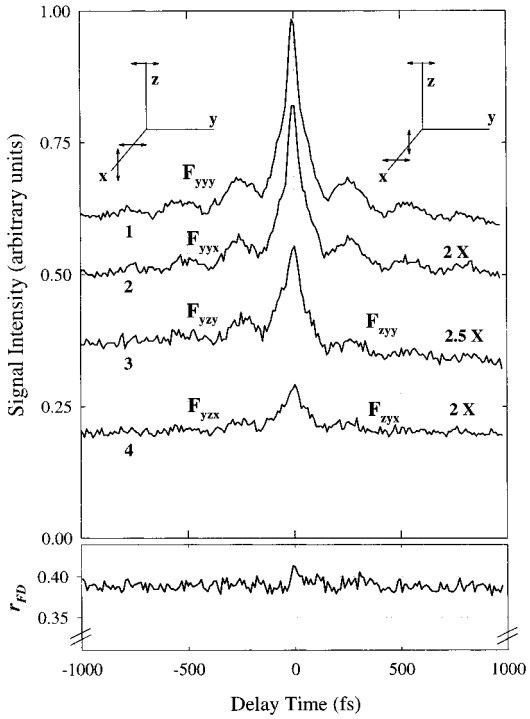

Figure 1. (top) Traces 1 and 2 are fluorescence-depletion signals for IR132 in DMSO when pulses *a* and *b*, propagating along the laboratory *x* axis, are polarized parallel to *y*; traces 3 and 4 are the signals when *b* has *z* polarization. Traces 1 and 3 were measured with the detection polarizer parallel to *y*, 2 and 4 with detection parallel to *x*. The insets depict the coordinate system and polarizers for trace 3. (bottom) Anisotropy signal calculated from the traces in the top panel.

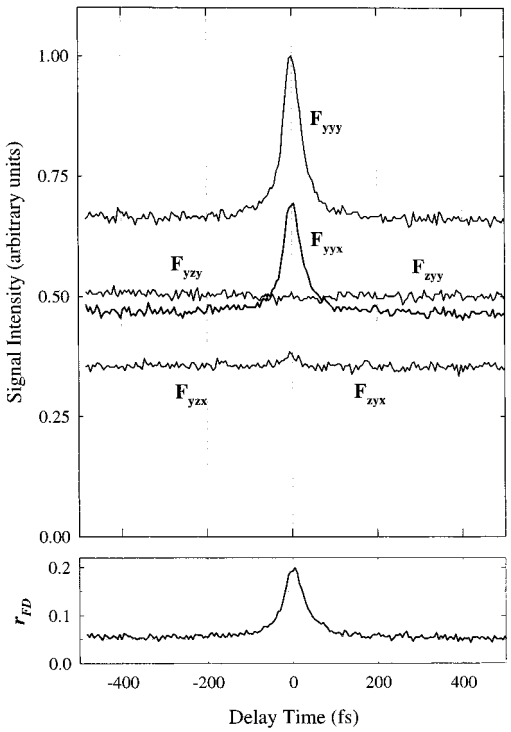

Figure 2. Similar to Figure 1, but for detergent-solubilized LH2 complexes.

complexes. To decrease light scattering, the membranes were treated with 0.1% lauryldimethylamine oxide, 20 mM Tris HCl, pH 8, and clarified by centrifugation. The measurements were repeated 3 times on different days with essentially identical results.

Results

Figure 1 shows fluorescence-depletion signals measured with the four polarization combinations for IR132. In traces 1 and 2, both excitation pulses are polarized along the laboratory *y* axis and the detection polarizer is parallel to either *y* or *x*. The total fluorescence-depletion signal is $F_{||} = F_{yyy} + 2F_{yyx}$, where the three subscripts indicate the polarizations of the excitation pulses in chronological order, followed by the detection. (Note that because of the axial symmetry of the excitation, $F_{yyz} = F_{yyx}$.) For identical pulses, F_{yyy} and F_{yyx} should have even symmetry around $\Delta t = 0$. Symmetric signals also are expected if the two pulses have perpendicular polarizations and the detection polarizer is orthogonal to both, as in trace 4 ($F_{yzx} = F_{zyx}$). An asymmetric signal can be obtained if the detection polarizer is parallel to the polarization of one pulse (say, pulse *a*) but perpendicular to that of the other, as in trace 3. In this case, if the anisotropy decay time is comparable to or longer than the fluorescence lifetime, the detector preferentially sees fluorescence from molecules that are excited by pulse *a*. If the fluorescence depletion caused by SE plus ESA differs from the depletion caused by GSB, the signal will depend on whether *a* arrives before or after *b*. However, a 90° rotation of the coordinate system around the *x* axis shows that $F_{zyy} = F_{yzz}$ and $F_{zyz} = F_{yzy}$. The total fluorescence-depletion signal for perpendicular excitation pulses thus is $F_{\perp} = F_{zyx} + F_{zyy} + F_{zyz} = F_{zyx} + F_{zyy} + F_{yzy} = F_{yzx} + F_{yzy} + F_{yzz} = F_{yzx} + F_{yzy} + F_{zyy}$, which is the sum of the signal measured with *x*-polarized detection and twice the time-symmetrized signal with *y*-polarized detection. The fluorescence-depletion anisotropy is $r_{FD} = (F_{||} - F_{\perp})/(F_{||} + 2F_{\perp})$.

The anisotropy signal $r_{FD}(\Delta t)$ thus determined for IR132 is shown in Figure 1 (bottom). The anisotropy is essentially constant between 0 and 1 ps, with a value of 0.39 ± 0.005, very close to the expected value of 0.4 for a molecule with parallel absorption and emission transition dipoles that are immobile on this time scale. The time-averaged conventional fluorescence anisotropy (r_F) can be obtained from the same measurements by calculating the parallel and orthogonal emission signals with pulse *b* rendered unpolarized. The appropriate combinations for this are $F_{yyy} + 2F_{yzy}$ and $F_{yyx} + F_{zyy} + F_{yzx}$ for parallel and orthogonally polarized detection, respectively. This procedure gave $r_F = 0.22$.

For measurements on LH2 complexes, chromatophore membranes were solubilized with a detergent to reduce light scattering. The background signals from the solubilized complexes, measured with one or the other pulse blocked, were less than 1% of the asymptotic depletion signals. Figure 2 shows representative signals. All the individual signals are nearly symmetric around zero time. This is expected, because measurements of fluorescence and time-resolved absorption have shown that the anisotropy decays to ~0.1 within 200 fs, which is much less than the excited-state lifetime.[4,7] The anisotropy obtained by the present technique peaks at zero time and decays rapidly to ~0.055 (Figure 2, bottom). The time-averaged r_F was 0.08 ± 0.005, which is close to the value of ~0.07 measured at 4.2 K.[8]

Discussion

Time-resolved fluorescence depletion has been used previously to study rotational spectra and vibrational relaxation dynamics in the gas phase.[9] More recently, two-color fluorescence depletion was used to study vibrational relaxations of

molecules in solution.[10] Kuśba et al.[5] analyzed the emission anisotropy of a sample after the action of two pulses, but did not measure the anisotropy decay by varying Δt as described here.

In a two-level system, the time-dependent fluorescence depletion is expected to be very similar to the time-resolved transient absorption. The "dump" or "probe" pulse both deexcites the emitting state and excites a ground state whose population has been reduced by the "pump" pulse. We have simulated the femtosecond absorption spectra of IR132 in DMSO by starting from an assumed energy autocorrelation function.[11] The same autocorrelation function with no additional adjustable parameters gives a predicted pump−dump signal very similar to the experimental F_{yyy} in Figure 2 (not shown). The calculated signal has a somewhat larger spike at $\Delta t = 0$ but reproduces the slower parts of the decay and the size of the quantum beats well. The pump−dump method thus has no particular advantage over pump−probe in a two-level system.

In LH2 and other systems with three or more levels, fluorescence-depletion anisotropy and absorption anisotropy contain slightly different information. For a well-separated pair of pulses, the fluorescence-depletion signal is given by

$$I_{FD}(\Delta t) = -C\{f_{gsb}(\Delta t) + f_{se}(\Delta t) + f_{esa}(\Delta t)[1 - p]\}$$

where C is a constant, f_{gsb} represents the depletion of S_0 by the first pulse, f_{se} and f_{esa} are the populations that the second pulse deexcites from S_1 back to S_0 or promotes to S_n, respectively, and p is the probability that a molecule raised to S_n returns to S_1. Depending on the value of p the term $f_{esa}(\Delta t)[1 - p]$ can either *add* to the depletion signal ($p \sim 0$) or make no significant contribution ($p \sim 1$). Contrast this to one-color pump−probe spectroscopy, where the sign of the ESA signal is opposite that of SE and GSB. In principle, the contributions of ESA to the depletion and pump−probe signals can be determined by comparing the anisotropies measured in the two experiments. The time-dependent fluorescence depletion (FD) and pump−probe (PP) anisotropy signals expressed in terms of the individual constituents are

$$r_{FD}(\Delta t) = f_{gsb}r_{gsb}(\Delta t) + f_{se}r_{se}(\Delta t) + f_{esa}r_{esa}(\Delta t)[1 - p]$$

$$r_{PP}(\Delta t) = \phi_{gsb}r_{gsb}(\Delta t) + \phi_{se}r_{se}(\Delta t) - \phi_{esa}r_{esa}(\Delta t)$$

with the normalizations $f_{gsb} + f_{se} + f_{esa}[1 - p] = 1$ and $\phi_{gsb} + \phi_{se} - \phi_{esa} = 1$. If the fractional contributions are known, it should be possible to evaluate $r_{esa}(\Delta t)$ and p by comparing the two measurements.

The anisotropy decay near $\Delta t = 0$ has contributions from coherent interactions of the two pulses with the sample, in addition to relaxations and dephasing of the excited complex, all convoluted with the cross correlation of the frequency-chirped pulses.[4] Because of its complexity, we will ignore the initial anisotropy here and focus on the asymptote at $\Delta t \approx 1$ ps. In three measurements, the asymptotic r_{PD} was 0.055 ± 0.005. Previous broadband pump−probe measurements gave $r_{PP} \sim 0.1$ at ~150 fs.[4] The fractional contributions of GSB, SE, and ESA (f_{gsb}, f_{se} and f_{esa}) were estimated from the asymptotic difference absorption spectrum and the spectra of the dump and probe pulses. The measured difference spectrum (Figure 3 of ref 4) was fit to the sum of four Gaussian bands (two positive amplitudes and two negative), which were taken to represent the spectra of GSB, SE and two ESA bands. Because the pulses used here were spectrally narrower than those in the pump−probe experiments, the fractional contributions of ESA and SE were smaller here. The values obtained were $f_{gsb} = 0.47$, $f_{esa} = 0.48$, $f_{se} = 0.05$, $\varphi_{gsb} = 4.45$, $\varphi_{esa} = -4.13$, and $\varphi_{se} = 0.68$. Because this leaves four unknowns and only two equations, we can only parametrize the solution in r_{se} and p. Figure 3 shows the possible values of r_{esa} and r_{gsb} as functions of r_{se} for the limiting cases $p = 1$ and 0. The measured time-averaged emission anisotropy (vide supra) sets a lower limit of 0.08 for r_{se}. The possible anisotropies of ESA and GSB are lower for $p = 1$ than for $p = 0$.

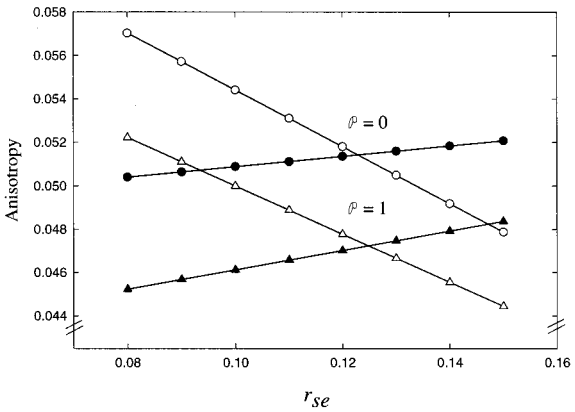

Figure 3. Anisotropies r_{gsb} (open symbols) and r_{esa} (closed symbols) calculated as a function of r_{se} for $p = 0$ (circles) and $p = 1$ (triangles).

This analysis assumes that the samples studied by the two techniques were identical in all important details, which may not have been the case. As noted above, the samples studied here were solublized with a detergent, whereas the pump−probe studies used intact membranes. In membranes, migration of excitons over many different LH2 complexes could decrease the anisotropy of SE and ESA to zero. However, this process occurs mainly on the time scale of 10 ps or longer,[12] and should have relatively little effect at the shorter times studied here.

From the crystal structure of the LH2 complex, the anisotropies of ESA, GSB, and SE are all predicted to be ~0.1 after dephasing and thermalization of a singly-excited complex.[4] The values of r_{gsb} and r_{esa} in Figure 3 are smaller than this, suggesting that our understanding of the spectroscopic properties of the LH2 complex is incomplete. The discrepancy is worse with $p = 1$ than with $p = 0$. If we take this to mean that p is closer to zero than to 1, the higher states created by ESA evidently do not regenerate S_1, but rather decay mainly by some other route, either nonradiatively or with emission of light outside our detection band (860−900 nm).

Fluorescence quenching at high excitation intensities has been studied extensively in chromatophores as a means of determining the "domain size" of the interconnected antenna network.[13] Quenching of fluorescence due to excitation annihilation increases with the excitation density. The quenching often is assumed to involve energy transfer from one excited molecule to another followed by radiationless decay of the multiply-excited molecule back to S_1, which would leave one molecule singly excited and the other unexcited.[14] This sequence, however, would not cause fluorescence depletion in the present experiments. Our results favor a process in which S_n decays predominantly to nonfluorescent states.

Femtosecond fluorescence-depletion anisotropy appears to be a promising approach for disentangling the contributions of ESA, SE, and GSB in complex systems and for exploring the fates of higher excited states. Future experiments will employ spectrally narrower pulses to probe ESA and SE more selectively.

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