

WAVELENGTH-SELECTIVE LIGHT QUENCHING OF BIOCHEMICAL FLUOROPHORES

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ABSTRACT

The use of light quenching to selectively eliminate the emission of biochemical fluorophores based on the emission wavelength is described. To demonstrate the possibility of wavelength-selective light quenching, a mixture of two fluorophores, 4-(dimethylamino)-4'-cyanostillene (DCS) and Prodan, emitting at different wavelengths was examined first. The emission spectrum and intensity decay were altered by the 570-nm quenching pulse due to selective quenching of the longer wavelength emission of DCS. Quenching of the solvent-sensitive fluorophore partially bound to human serum albumin and partially in the aqueous phase was then examined. Light quenching with a long wavelength (570 nm) time-delayed pulse selectively quenched the Prodan fluorophore in the aqueous phase while in the presence of the Prodan bound to human serum albumin, which emitted at shorter wavelengths, was not quenched. Using one-beam short wavelength excitation and quenching, a selective quenching of the blue-shifted emission of ribonuclease T_1 in the presence of the red-shifted emission of the tryptophan residue in adrenocorticotrophic hormone was observed. In both systems wavelength-selective light quenching was demonstrated by a shift in the emission spectra, and by changes in the intensity decay consistent with preferential quenching of one species. Light quenching is instantly reversible by blocking or defocusing of the quenching beam. It can occur for inaccessible residues or in viscous solvents, and thus can be of wide applicability for resolving the complex emission of biological macromolecules. © 1997 Society of Photo-Optical Instrumentation Engineers.

Keywords wavelength-selective light quenching, time-resolved fluorescence, biophysics.

1 INTRODUCTION

Quenching of fluorescence is widely used in biochemical research as a method for studying the structure and dynamics of macromolecules.¹ A wide variety of substances are known to act as collisional quenchers, including oxygen, iodide, halogens, amines, and acrylamide, to name a few.^{1,2} Collisional quenching has been used to reveal the rates of diffusion of quenchers in proteins³⁻⁶ and in membranes,⁷⁻⁸ and to determine the relative exposure of fluorophores in proteins and membranes to the aqueous phase or to the internal region of the macromolecules.⁸⁻¹¹ An additional use of collisional quenching is to control the excited-state lifetime, and thereby alter the time available for rotational diffusion or spectral relaxation.¹² Such lifetime-resolved measurements have been used to quantify rotational diffusion and time-dependent spectral shifts of labeled membranes¹³⁻¹⁴ and segmental motions of tryptophan residues in proteins.¹⁵⁻¹⁶ The occurrence of significant collisional quenching requires that the media or macromolecule be adequately fluid for translational motion during the excited-state lifetime and that the fluorophore be accessible to the quenchers. In addition, one must

consider the possibility that the quenchers interact with the macromolecules and thereby alter their properties. In spite of these limitations, fluorescence quenching is a powerful method for studying the structure and dynamics of biological macromolecules.

We now describe the use of light itself as a quencher of fluorescence. In recent reports we have shown that illumination of samples with wavelengths overlapping the emission spectra can result in quenching by the process of stimulated emission. Since the samples are observed 90 deg to the incident light, the observed intensity is decreased, and we refer to this phenomenon as light quenching.¹⁷ The extent of light quenching depends on overlap of the emission spectra with the quenching wavelength, the time delay between the excitation and quenching pulses, and on the orientation of the fluorophore relative to the electric vector of the quenching beam.¹⁸⁻²¹ The occurrence of light quenching with high repetition-rate ps dye lasers has been demonstrated by both steady-state¹⁸⁻²¹ and time-resolved measurements.²²

In this article we show that wavelength-selective light quenching can be used to preferentially deplete the emission of one fluorophore in a mixture

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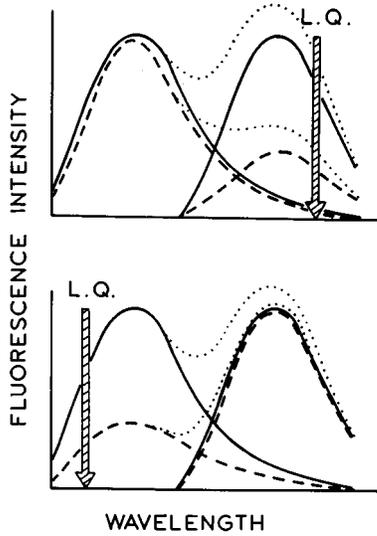


Fig. 1 Intuitive description of wavelength-selective light quenching. The solid and dashed lines show the emission spectra of particular components in the presence and absence of light quenching, respectively. The dotted lines illustrate the respective spectra emitted by the mixture of the components.

of fluorophores. The nature of these experiments is shown in Figure 1. Suppose the emission is due to two fluorophores, which emit at overlapping but different wavelengths. Illumination of the sample with long wavelengths, overlapping the emission of the longer wavelength fluorophores, is expected to preferentially quench this species. In this case long wavelength quenching is expected to result in a blue shift in the steady-state emission spectrum. Conversely, illumination on the short wavelength side of the emission is expected to quench the shorter wavelength fluorophore, resulting in a red shift in the emission spectrum. The possibility of wavelength-selective light quenching was demonstrated first for a mixture of fluorophores emitting at different wavelengths. We then determined that the emission of an aqueous phase fluorophore can be quenched in the presence of the same fluorophore bound to a protein. Finally we show preferential quenching of a buried tryptophan residue in the presence of a solvent-exposed tryptophan residue.

2 THEORY

We will consider two types of light quenching experiments. In a "one-beam" experiment, excitation and emission are due to the same light beam, and occur simultaneously during the excitation pulse. In this case the excitation and quenching wavelengths are identical. In a "two-beam" experiment, excitation and quenching are due to different light beams, which are typically of different wave-

lengths. In this case the first pulse excites the sample, and the second pulse quenches the emission.

The theory for time-resolved measurements is somewhat different for one-beam and two-beam light quenching, and a complete description will be presented elsewhere.²³ In the case of one-beam light quenching, the intensity decay of the two-fluorophore mixture can be expressed as

$$I(t) = J \left[\frac{f_1}{\tau_1} \exp(-t/\tau_1) + \frac{f_2}{\tau_2} \exp(-t/\tau_2) \right], \quad (1)$$

where τ_k are the respective fluorescence lifetimes, J is the steady-state emission intensity of the mixture, and f_k are the fractions of fluorescence contributed by the fluorophores to the steady-state emission. In the case of two-beam light quenching, the intensity decay displays an instantaneous jump at time $t = t_d$ describing the time of the arrival of the quenching pulse.^{17,22} The intensity decreases may be different for each fluorophore and are described by parameters q_k

$$q_k = \frac{I_{kb} - I_{ka}}{I_{kb}}, \quad (2)$$

where I_{kb} and I_{ka} are the fluorescence intensities of the respective fluorophores immediately before and after the quenching pulse. The expression for the intensity decay in the presence of two-beam light quenching takes the form

$I(t)$

$$= \begin{cases} J \left[\frac{f_1}{Z_1} \exp(-t/\tau_1) + \frac{f_2}{Z_2} \exp(-t/\tau_2) \right], & 0 \leq t \leq t_d \\ J \left[\frac{f_1}{Z_1} (1 - q_1) \exp(-t/\tau_1) + \frac{f_2}{Z_2} (1 - q_2) \right. \\ \quad \left. \times \exp(-t/\tau_2) \right], & t > t_d. \end{cases} \quad (3)$$

where Z_k are the normalization factors defined as

$$Z_k = \tau_k [1 - q_k \exp(-t_d/\tau_k)]. \quad (4)$$

We examined the intensity decays of the mixture, with and without light quenching, using the frequency-domain method. For the analysis, the frequency responses are calculated from Eqs. (1) and (3) at each modulation frequency (ω) using the two frequency-dependent quantities.^{24,25}

$$N_\omega = \int_0^\infty I(t) \sin(\omega t) dt, \quad (5)$$

$$D_\omega = \int_0^\infty I(t) \cos(\omega t) dt. \quad (6)$$

These quantities were then used to find the calculated phase angle ($\phi_{c\omega}$) and the calculated luminescence modulation ($m_{c\omega}$)

$$\phi_{c\omega} = \arctan(N_{\omega}/D_{\omega}), \quad (7)$$

$$m_{c\omega} = \frac{1}{J} (N_{\omega}^2 + D_{\omega}^2)^{1/2}. \quad (8)$$

The best-fitted parameters, f_k and q_k , and goodness of fit were determined by the minimum value of

$$\chi_R^2 = \frac{1}{\nu} \sum_{\omega} \left[\left(\frac{\phi_{\omega} - \phi_{c\omega}}{\delta\phi} \right)^2 + \left(\frac{m_{\omega} - m_{c\omega}}{\delta m} \right)^2 \right], \quad (9)$$

where ϕ_{ω} and m_{ω} are the experimental phase and modulation, respectively, $\delta\phi = 0.2$ deg and $\delta m = 0.005$ are the experimental uncertainties, and ν is the number of degrees of freedom.

3 MATERIALS AND METHODS

3.1 ONE-BEAM LIGHT QUENCHING

The experimental arrangements are different for one-beam and two-beam light quenching. We used one-beam light quenching to study selective quenching of tryptophan residues in RNase T_1 and Adrenocorticotrophic hormone (ACTH). For the one-beam experiments, the excitation and light quenching are due to the same incident beam. In order to obtain overlap of the excitation with the emission spectrum, it is necessary to excite the sample on the extreme long wavelength edge of the absorption spectra. We used an experimental arrangement similar to that used previously for one-beam light quenching¹⁸ except that the light source was a frequency-doubled and cavity-dumped rhodamine 6G dye laser at 300 nm. The dye laser was synchronously pumped by the 514-nm output of a mode-locked argon ion laser. The pulse width was near 5 ps, the repetition rate was 3.795 MHz, and the average power was 5 mW. This light was focused to a spot size about 20 μm in diameter, resulting in a maximum intensity of about 2×10^3 W/cm². Without focusing, the spot size was about 2 mm in diameter. The emission was selectively observed from the focal region of the illuminated sample using a spatial filter 3 mm high and 200 μm wide.

The excitation was polarized vertically, as occurs from the output of our dye laser. The emission was observed through a Corning filter, which transmits light above 320 nm. For intensity measurements, the emission polarizer was 54.7 deg from the vertical. Control measurements using the buffer without the proteins gave signals less than 0.5% of the protein emission, for all polarization conditions and excitation (quenching) wavelengths.

Ribonuclease T_1 was a gift from Dr. Maurice Eftink, University of Mississippi, and the ACTH was obtained from Ciba-Geigy. The proteins were

dissolved in 0.1 M sodium acetate, pH 5.5. For measurements of each protein alone, the concentrations were near 2×10^{-5} M. In the mixture of RNase T_1 and ACTH, the concentrations were 1.1 and 1.3×10^{-5} M, respectively.

3.2 TWO-BEAM LIGHT QUENCHING

Two-beam light quenching was used for studies of the DCS-Prodan mixture, and for light quenching of free and human serum albumin (HSA)-bound Prodan. The experimental arrangement for two-beam light quenching was the same as described previously.^{21,22} The light source was again the R6G dye laser, but in this case the frequency-doubled output at 285 nm was used for excitation, and the fundamental output at 570 nm was used for light quenching. The average power at 285 and 570 nm was 5 and 150 mW, respectively. The region of overlap of the excitation and quenching beams was selectively observed with the 3 mm \times 200 μm spatial filter. The time delay between the excitation and quenching pulses was 20 ps for all experiments.

In the mixture of DCS and Prodan, the concentrations in the dimethylformamide (DMF) solvent were 2×10^{-5} M and 3×10^{-5} M, respectively. The human serum albumin (HSA) was in 20 mM phosphate buffer, pH 7.4, with an HSA concentration near 10^{-5} M and a Prodan concentration of 6×10^{-5} M.

Intensity and anisotropy decay measurements were obtained using frequency domain (FD) instrumentation described previously.²⁶⁻²⁷ For intensity decay measurements, the excitation was vertically polarized and the emission observed through a polarizer 54.7 deg from the vertical. The FD intensity decay data were analyzed as described previously.²⁴⁻²⁵

4 RESULTS

4.1 TWO-BEAM LIGHT QUENCHING OF A FLUOROPHORE MIXTURE

To evaluate the prospects for wavelength-selective light quenching, we first examined a mixture of two fluorophores, DCS and Prodan. These fluorophores were selected for the possibility of excitation at the same wavelength 285 nm, and for different fractional overlaps of their emission spectra with the 570-nm quenching wavelength (Figure 2). In the DMF solvent, Prodan and DCS display emission maxima of 445 and 515 nm, respectively. The intensity decay of each fluorophore alone is nearly a single exponential, with decay times of 0.68 and 3.9 ns, respectively (not shown).

Examination of Figure 2 indicates that the emission spectrum of DCS overlaps more strongly with the quenching beam than does the emission of Prodan. Hence, one expects selectively more light quenching of DCS than Prodan for 570-nm quenching. The emission spectrum of the mixture was

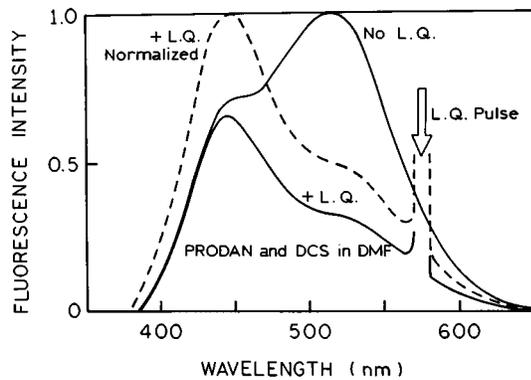


Fig. 2 Emission spectra of a mixture of Prodan and DCS in DMF.

found to be strongly blue shifted upon 570-nm illumination (Fig. 2, +LQ and dashed line). This effect was immediately reversible upon blocking of the quenching beam.

We also determined the extent of light quenching from the intensity decays measured in the frequency domain. Intensity decays were measured at 480 nm, where we expected comparable intensities from each fluorophore. In the absence of light quenching, the intensity decay is a double exponential [Figure 3(a), and Table 1], as is expected for a mixture of two fluorophores, each of which displays a single exponential intensity decay. In the presence of light quenching, the intensity decay remains a double exponential. However, the shape of the frequency response is altered, as can be seen from the comparison of the data with and without light quenching in Fig. 3(b). Analysis of these data indicates that the decay times were unchanged by light quenching, but the fractional intensity of each fluorophore was altered by light quenching. To be specific, the intensity of DCS was quenched by 68.5%, while the intensity of Prodan was only quenched by 3.4% (Table 1). Without light quenching, the fractional intensities of DCS and Prodan are 0.75 and 0.25, respectively. With light quenching, the respective fractional intensities are nearly equal—0.51 and 0.49. The higher fractional contri-

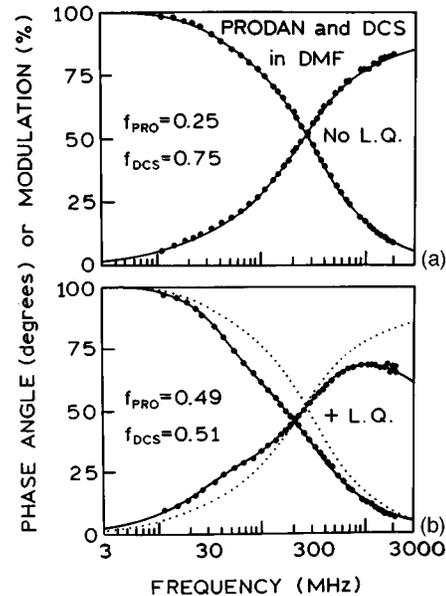


Fig. 3 Frequency-domain intensity decay of a mixture of Prodan and DCS in (a) the absence and (b) the presence of light quenching. The solid lines show the best fit to the experimental data (●) based on Eq. (3) for $\tau_1 = \tau_{DCS} = 0.68$ ns and $\tau_2 = \tau_{PRO} = 3.9$ ns. The dotted line represents the frequency response in the absence of light quenching (shown by the solid line in the upper panel).

bution of the longer lifetime Prodan can be seen in the FD data by the higher phase angles and lower modulation values from 30 to 100 MHz. These data show that the selectivity of light quenching can be quite high, in this case DCS being quenched 20-fold more strongly than Prodan.

In a previous report we described oscillations in the frequency-domain data due to time-delayed light quenching.²² Hence, one may question the absence of oscillations in Fig. 3. The oscillations are not visually apparent because of the short decay time of 20 ps. The oscillations are just becoming apparent in the data above 1 GHz (Figure 3).

Table 1 Intensity decays in the absence and presence of long-wavelength two-pulse light quenching.

Sample	Quenching	f_1	f_2	q_1	q_2	χ_R^2
DCS ($\tau_1 = 0.68$ ns) and Prodan	No LQ	0.749	0.251	0	0	1.2
($\tau_2 = 3.9$ ns) in DMF	+LQ	0.506	0.494	0.685	0.034	1.2
Prodan ($\tau_1 = 0.57$ ns) and Prodan-HSA	No LQ	0.858	0.142	0	0	1.3
($\tau_2 = 3.7$ ns)	+LQ	0.664	0.336	0.672	0.035	0.8

Note: Intensity decays were measured at 480 nm. Values of the lifetimes τ_1 and τ_2 are those used in Eq. (3) and refer to both the absence and presence of light quenching. These lifetimes were held fixed during the analysis.

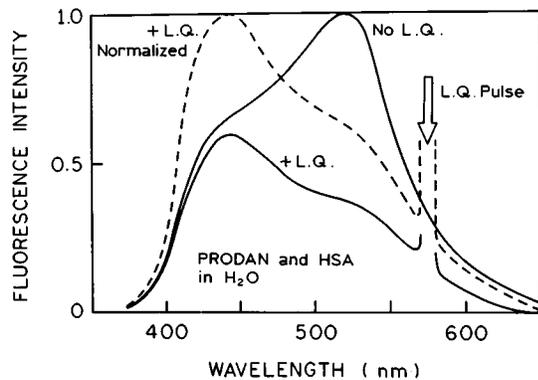


Fig. 4 Emission spectra of Prodan partially bound to HSA.

4.2 TWO-BEAM LIGHT QUENCHING OF PRODAN IN SOLUTION AND BOUND TO HSA

We next considered the possibility of selective light quenching of the same fluorophore, Prodan, in two different environments. In water, Prodan displays a red-shifted emission with a maximum near 510 nm (Figure 4). Upon binding to HSA, the emission of Prodan is strongly blue shifted to an emission maximum near 445 nm. Based on these spectral properties, we expected free Prodan to be more strongly quenched by 570-nm illumination.

Emission spectra of Prodan partially bound to HSA are shown in Figure 4. Upon illumination with 570 nm, the emission spectrum is strongly blue shifted, which is consistent with preferential quenching of free Prodan. We also measured the frequency-domain intensity decay of Prodan. In water, and when completely bound the HSA, the intensity decays are reasonably single exponentials with decay times of 0.57 and 3.7 ns, respectively (not shown). When Prodan is partially bound to HSA, the intensity decay of the solution is a double exponential (Figure 5 and Table 1). In the absence of light quenching, the time-resolved emission is mostly due to free Prodan, which displayed a fractional intensity of 0.858. Upon 570-nm light quenching, the fractional intensity of free Prodan decreased to 0.664 (Table 1). The higher contribution of HSA-bound Prodan to the time-resolved emission can be seen from the increase in phase angle and decrease in modulation near 50 MHz (Figure 5). The decrease in phase angle above 500 MHz in the presence of light quenching (Figure 5) is due to the onset of oscillations in the FD data caused by the time-delayed quenching pulse.²²

4.3 ONE-BEAM LIGHT QUENCHING OF THE BURIED TRYPTOPHAN RESIDUE IN RIBONUCLEASE T_1

In the previous section we demonstrated that light quenching could preferentially quench a fluorophore in the aqueous phase, which is also possible

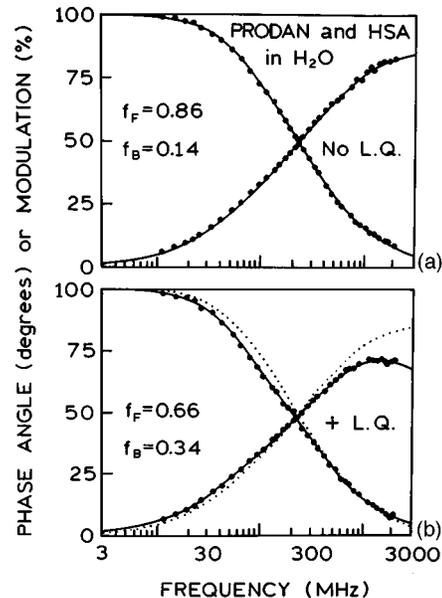


Fig. 5 Frequency-domain intensity decay of Prodan-HSA, in (a) the absence and (b) the presence of light quenching. The solid lines show the best fit to the experimental data (●) based on Eq. (3) for $\tau_1 = \tau_F = 0.57$ ns and $\tau_2 = \tau_B = 3.7$ ns. The dotted line represents the frequency response in the absence of light quenching (shown by the solid line in the upper panel).

for collisional quenchers. Hence we questioned whether light quenching could preferentially quench a fluorophore shielded from the solvent. To answer this question, we examined a mixture of two proteins, ACTH and RNase T_1 , each of which contains a single tryptophan residue. The residue in ACTH is fully exposed to solvent,^{1,28} and displays a red-shifted emission [Figure 6(a)]. The tryptophan residue in RNase T_1 is completely shielded from the solvent,²⁸⁻²⁹ and displays a blue-shifted emission (Figure 6).

Because of limitations of our laser sources, we were not able to accomplish two-beam light quenching of proteins with a time-delayed pulse. Hence, we use one-beam light quenching in which the focused excitation pulse both excites the fluorophores and causes light quenching. Excitation and quenching were accomplished using 300-nm excitation, which overlaps with the emission of RNase T_1 but not with the emission of ACTH. In the mixture of RNase T_1 and ACTH without light quenching, the emission spectrum shows a dominant contribution of RNase T_1 [Figure 6(b)]. In the presence of light quenching, the emission maximum shifts to longer wavelengths, demonstrating that the buried tryptophan in RNase T_1 is quenched more strongly than the exposed residue in ACTH. Preferential quenching of a buried residue is the opposite of that usually observed with collisional quenchers, which illustrates the complementary characteristics of light quenching and collisional quenching.

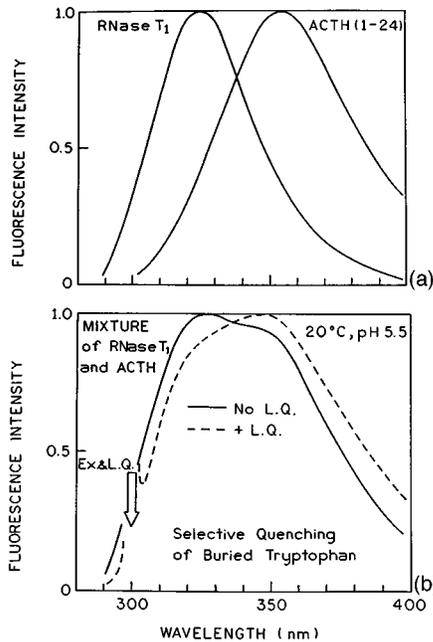


Fig. 6 Emission spectra of (a) RNase T_1 and ACTH and (b) a mixture of RNase T_1 and ACTH with (---) and without (—) light quenching.

We also examined how the intensity decays of the protein mixture have affected by light quenching. The intensity decay of RNase T_1 alone is a single exponential of 3.85 ns.^{28–29} The intensity decay of ACTH is not a single exponential, and requires at least three decay times to describe its intensity decay.²⁹ However, for simplicity we treated the intensity decay of ACTH as a single exponential with a mean decay time of 2.36 ns. Frequency-domain intensity decay data for a mixture of RNase T_1 and ACTH are shown in Figure 7. The intensity decay in the mixture could not be fit to the single exponential model (Table 2). However, the double exponential model gave a reasonably good fit. Without light quenching, the double exponential analysis

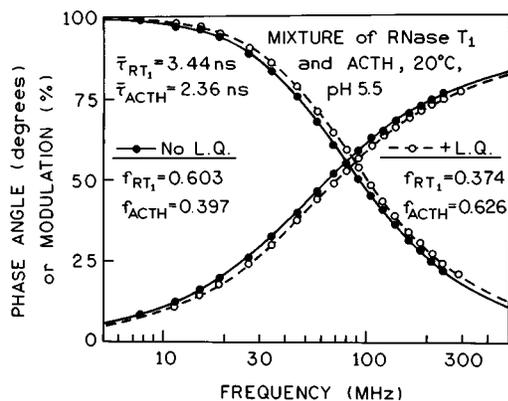


Fig. 7 Frequency response of a mixture of RNase T_1 and ACTH without (—●—) and with (---○---) one-beam light quenching.

Table 2 Intensity decays of ACTH and RNase T_1 , in the absence and presence of short-wavelength one-pulse light quenching.

Sample	Quenching	f_1	f_2	χ_R^2
RNase T_1 ($\tau_1=3.44$)	No LQ	1.0	-	2.1
ACTH ($\tau_2=2.36$)	No LQ	-	1.0	79.4
RNase T_1 ($\tau_1=3.44$) and ACTH ($\tau_2=2.36$)	No LQ	1.0	-	7.2 ^a
	+LQ	0.37	0.63	2.1

^a The apparent single decay time was 2.92 ns. Values of the lifetimes τ_1 and τ_2 are those used in Eq. (1) and refer to both the absence and presence of light quenching. These lifetimes were held fixed during the analysis.

yields decay times of 3.44 and 2.36 ns with fractional intensities of $f_1=0.603$ and $f_2=0.397$ for RNase T_1 and ACTH, respectively (Table 2). As predicted from the emission spectrum, the emission of RNase T_1 is dominant without light quenching. In the presence of light quenching, the frequency response of the mixture shifts to higher frequencies, indicating an increased contribution of the ACTH. Light quenching occurs preferentially for RNase T_1 , so that the emission of ACTH becomes the major component in the intensity decay in the presence of light quenching ($f_2=0.625$). These results demonstrate the possibility of wavelength-selective light quenching of tryptophan residues in proteins.

5. DISCUSSION

What are the advantages of using light as a quencher, compared with the usual use of collisional quenchers added to the solution of macromolecules.² One obvious advantage is the immediate reversal of quenching by blocking or defocusing the incident beam. Small amounts of light quenching can thus be detected using an optical chopper and lock-in detection methods. In contrast, collisional quenchers must be removed by dialysis or chromatographic methods.

An additional advantage of light quenching is that it can occur in highly viscous solutions that do not allow significant translational diffusion during the excited-state lifetime. Since light quenching depends on spectral overlap, and not physical contact between the fluorophore and quencher, light quenching can be expected to occur for fluorophores that are not accessible to collisional quenchers, as was shown for RNase T_1 . Depending on the emission spectra and quenching wavelength, light quenching can occur for either solvent-exposed or for buried residues. In contrast, significant collisional quenching only occurs in low-viscosity conditions, and requires molecular contact between the fluorophore and quencher.

Light quenching also depends on the lifetime of the excited state. If the solution contains two fluorophores with different decay times, the extent of quenching of each fluorophore depends on its excited state population upon arrival of the quenching pulse. This property of light quenching may be especially valuable in studies of multichromophore systems, such as photosynthetic reaction centers or phycobiliproteins,^{30–32} in which energy is transferred between several chromophores. Examination of the emission properties with various time delays may provide information about the energy transfer process.

It should also be noted that light quenching is not expected to be photodestructive. Since light quenching returns the fluorophores to the ground state more rapidly than expected for the spontaneous decay, one can expect the fluorophore to be protected from photochemical processes by light quenching. Such protection against photochemical degradation has been used as a basis for measuring energy transfer efficiencies in fluorescence microscopy.^{33–34}

Finally, light quenching differs from collisional quenching in that light quenching depends on the orientation of the fluorophore relative to the polarized quenching beam. In contrast, there is no known dependence of collisional quenching on orientations. These differences between collisional quenching and light quenching indicate that the methods are complementary, and in combination can provide additional information about biological macromolecules.

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