Luminescence Quenching by Nitroxide Spin Labels in Aqueous Solution: Studies on the Mechanism of Quenching†

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ABSTRACT: The mechanism of luminescence quenching by spin labels was investigated in aqueous solution by steady-state and time-resolved luminescence techniques. Water-soluble nitroxide radicals strongly quenched the luminescence emitted by Tb^{3+} chelates and by fluorescein, either free or conjugated to proteins. The following features of the quenching reaction were established: (I) the rate constant for quenching of triplet-state Tb^{3+} by nitroxides was about 4 orders of magnitude smaller (ca. 10^5 M^{-1} s^{-1}) than those of the singlet-state probes; (II) the quenchers reduced the excited-state lifetime of both probes; (III) the rate constants for quenching of both probes were found to be apparently independent of the temperature (between 6 and 42 °C) and viscosity (up to 60 mPa·s) of the solutions; (IV) both singlet and triplet quenching rates were sensitive to solvent polarity; (V) there is a small but significant spectral overlap between the absorption band of weekly absorbing nitroxide radicals and the emission spectra of luminophores, the extent of which, however, does not correlate with the extent of quenching; (VI) the quenching rate declines sharply with an increasing luminophore to nitroxide distance. The distance dependence of the quenching rate showed a satisfactory fit to an exponential function. These findings indicate that the quenching reaction is dominated by an electron exchange between the excited singlet- or triplet-state luminophore and the nitroxide radical rather than controlled by diffusional properties of the reactants. The extremely high sensitivity of the quenching rate to the separation distance in the range of 0.5–2 nm should make nitroxide-conjugated antibodies paired with fluorophore-conjugated antibodies a useful fluorophore–quencher system for studying lateral organization and association of membrane proteins on cell surfaces.

It was recognized many years ago that some molecular species with electron spin multiplicities >0 are good quenchers for the excited electronic states of a wide variety of fluorescent molecules (Birks, 1970; Jost et al., 1971). These radicals, mostly in the form of spin-labeled phospholipids or fatty acid derivatives, are extensively used to study the distribution, location, and dynamics of proteins and lipids as well as their interaction in model and biological membranes [e.g., Bieri and Wallach (1975), London and Feigenson (1981a,b), London (1982), Blatt and Sawyer (1985), Chattopadhyay and London (1987), and Yeager and Feigenson (1990)].

In most of these studies with spin-labeled phospholipids, it was assumed that the radicals quench upon contact (Green et al., 1973; London & Feigenson, 1981a) and the quenching occurs at the diffusion limit (Chattopadhyay et al., 1983). Assumption of a mechanism for the fluorescence quenching strongly affects interpretation of experimental data on the mobility, depth, and separation of membrane components. The mechanistic details of the quenching interaction between singlet and triplet luminescent probes and water-soluble spin labels have not been resolved yet.

Earlier studies of the quenching mechanism concentrating mainly on possible quenching pathways in different solvent systems led to somewhat contradictory conclusions about contribution of different energy-exchange mechanisms to the observed quenching [see, e.g., Green et al. (1973), Kuzmin and Tatikolov (1977), Puskin et al. (1981), London (1982), and Green et al. (1991)], and no systematic analysis of the intermolecular quenching has been done so far with water-soluble quencher derivatives. Thus, in order to see what kind of molecular interactions dominate in the observed fluorescence quenching, we have analyzed the quenching of luminescence from Tb^{3+} chelates and fluorescein conjugates in aqueous solution.

This paper reports time-resolved and steady-state data on the quenching of luminescence of singlet probes fluorescein and sulfo-Rhodamine 101 as well as of triplet-state Tb^{3+} chelates using neutral and charged TEMPO derivatives as water-soluble quenchers. The quenching interaction was studied with free probes and small chelates as well as with probes conjugated to proteins (monoclonal antibody molecules). The data argue strongly against a diffusion-controlled collisional mechanism of the quenching, even in solution, and suggest that the quenching process is dominated by distance-dependent electron-exchange (transfer) interactions between excited-state fluorophores and radicals similar to the mechanism proposed by Green et al. (1990) for *intramolecular* quenching of excited singlet states in paramagnetic hydrocarbons. Our data also suggest that pairs of fluorophore- and spin-label-conjugated antibodies are potentially useful probes for the interactions of cell membrane proteins with one another.

Materials and Methods

Chemicals. Terbium(III) chloride ($TbCl_3$) hexahydrate (99.9%) was a product of Aldrich Chemical Co. (Milwaukee). Fluorescein isothiocyanate (FITC) and Texas Red sulfonyl chloride (TxR or sulfo-Rhodamine 101) were purchased from Molecular Probes (Eugene, Oregon). 3-[[2-(2-Isothiocyanatoethoxy)ethyl]carbamoyl]PROXYL (ITC-EECP) and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (4-hydroxy-TEMPO or TEMPOL) were obtained from Sigma Chemical Co. (St. Louis, MO). TEMPO-choline was a kind gift of B.

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(*S*)-4-[2,3-[Bis(carboxymethyl)amino]propyl]phenylisothiocyanate (CITC) (Meares et al., 1984) was kindly provided by Dr. Claude Meares of The University of California, Davis. Glycerol (Baker, spectroscopic grade), Dextran T-70 (Pharmacia, Uppsala, Sweden), PVP-370 [(polyvinyl)pyrrolidone] (Sigma), and sucrose (Baker Chemical) were used to raise solution viscosity. KI was also purchased from Baker. Fmoc–L-Pro–OPfp and Pepsyn KA with threonine were purchased from Milligen (MA). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide·HCl and fluoresceinamine (isomer I) were products of Sigma (St. Louis, MO). All the other chemicals were the highest purity grades available.

Conjugation of Antibodies with Fluorophores, Chelator, and Quenchers. A mouse monoclonal antibody KE-2, reactive with the class I major histocompatibility complex molecules of human cells (Wier & Edidin, 1986) was used throughout the quenching experiments. The purified IgG and/or the F_{ab} fragment obtained by papain digestion (Edidin & Wei, 1982) was labeled with isothiocyanate derivatives of either the Tb^{3+} chelator, fluorescein, or the spin-label quencher. The KE-2 antibody (ca. 2–3 mg/mL) was mixed with a 20–40-fold molar excess of a given isothiocyanate (CITC, FITC, or EECP-ITC) in 0.1 M sodium bicarbonate ($NaHCO_3$) buffer (pH 9.5) and incubated overnight at 4 °C. Excess free label was removed by gel filtration through a Sephadex G-50 column (1.2 × 30 cm) equilibrated with 10 mM Hepes buffer (pH 7.4, 145 mM NaCl, 5 mM KCl). The conjugation of the antibody with the chelator was checked by its Tb^{3+}-binding capacity detected by fluorescence measurements while the conjugation with EECP-ITC was followed by taking ESR absorption spectra of the conjugates. Labeling of KE-2 with fluorescein was monitored by measuring the absorbance of the conjugate at 280 and 494 nm. A molar extinction coefficient of 6.3×10^4 M^{-1} cm^{-1} at 494 nm (DePetris, 1978) was used for fluorescein conjugates. The specificity of the labeled antibodies was checked by competition with unlabeled antibodies on VA2 (transformed human fibroblast) and JY (human lymphoma) cells, and it was found to be unaltered by the labeling.

Sulfo-Rhodamine 101 was conjugated with L-lysine in 0.1 M $NaHCO_3$ (pH 9.0), applying a 5-fold molar excess of lysine. The conjugate was used after 1000-fold dilution into a 10 mM Hepes buffer (pH 7.4).

Synthesis and Labeling of Oligoproline Peptides. Oligoproline peptides (n = 1–10) each having one threonine amino acid at the carboxyl end [NH–$(Pro)_n$–Thr–COOH] were synthesized by the Fmoc-polyamide solid-phase peptide synthesis method (Carpino & Han, 1972; Atherton et al., 1978) using a Milligen 9050 peptide synthesizer. The resin was removed from the products by gentle shaking of the mixture with 95% trifluoroacetic acid and 5% phenol for 2 h at room temperature. The products were analyzed by HPLC (Waters). The peptides were then separated from the resin by filtration and a subsequent extraction by ether. The product was dried and washed twice with ether before use for labeling.

The aqueous solution of peptides [containing 30% (v/v) acetonitrile] was reacted with a 5-fold molar excess of fluoresceinamine in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (0.05 M) at pH 4.7, with gentle shaking for 12 h at room temperature. Then the pH of the reaction mixture was set to 9–9.5 with NaOH, 75 mM isothiocyanate derivative of the spin label was added, and the mixture was gently shaken for 48 h at 4 °C.

The reaction mixture was then analyzed by thin layer chromatography on fluorescent silica sheets using a mixture of chloroform/methanol/acetic acid/water as solvent (at a 25:15:4:2 volume ratio). Two major components were detected with R_f values of 0.88 and 0.27, respectively. The labeled and double-labeled peptides were separated from the unlabeled peptide, and the excess label was removed by gel chromatography through a Bio-Gel P-2 column (Bio-Rad) (15 × 0.8 cm). The fluorophore and nitroxide labeling of the peptide products were checked by spectrophotometry, fluorimetry, and ESR spectroscopy.

Spectroscopic Measurements. Fluorescence spectra and steady-state intensities were recorded in an SLM 8000 fluorimeter equipped with a thermostated, stirred cell holder and interfaced to an IBM AT microcomputer. The fluorescence intensities were corrected using a Rhodamine standard quantum counter and either a 5 mM Tb^{3+}/EDTA complex or a 0.5 μM fluorescein (pH 7.4) solution. The excitation wavelength for Tb^{3+} luminescence was 295 nm (indirect excitation) or occasionally 370 nm. The Tb^{3+} emission was collected from 520 to 570 nm, and the intensity values were derived by integrating the peak centered at 545 nm. An excitation wavelength of 485 nm was used for fluorescein conjugates, and the emission was collected from 500 to 560 nm. The intensities were derived from the peak centered around 520 nm. Fluorescence of sulfo-Rhodamine 101 was excited at 585 nm, and the emission was recorded between 590 and 640 nm. (The emission peak centered around 615 nm.) There was negligible light scattering from all samples. When necessary, the fluorescence intensities were corrected for the absorption increment at the excitation wavelength caused by added compounds or quenchers, as described earlier (Matko et al., 1980). The molar extinction coefficients for TEMPOL and TEMPO-choline at 485 nm were experimentally determined in 10 mM Hepes, pH 7.4, and found to be 6.7 M^{-1} cm^{-1} and 3.8 M^{-1} cm^{-1}, respectively. The temperature of the samples was controlled within ±0.1 °C.

Luminescence quenching data were obtained by recording the intensities after successive addition of small aliquots of quencher stock solutions. In case of quenching by iodide anions, the quencher stock solution contained 0.1 mM $Na_2S_2O_3$ to avoid I_3^- formation. The data were analyzed with a program on the Stern–Volmer theory and Smoluchowski's diffusion equation (Lakowicz, 1983). In case of labeled peptides, the extent of the quenching was determined as $1 - F_{fq}/F_f$, where F_{fq} and F_f are the corrected and normalized (to the same concentration) fluorescence intensities measured in double-labeled and fluorophore-labeled peptides, respectively.

Decay of Tb^{3+} luminescence was recorded by a phosphorescence spectrometer (laboratory of J. M. Vanderkooi, University of Pennsylvania, Philadelphia). Data collection and analysis were carried out as described elsewhere (Green et al., 1988). The decay of fluorescein emission was recorded on a 2-GHz frequency-domain fluorometer (Center for Fluorescence Spectroscopy, University of Maryland, Baltimore). Data were collected and analyzed as described (Lakowicz et al., 1984; Lakowicz & Maliwal, 1985). The ESR spectra of the spin labels and labeled antibodies were recorded on an ESR spectrometer in laboratory of B. Gaffney (Department of Chemistry, The Johns Hopkins University, Baltimore) at ca. 9-GHz microwave frequency using 100-kHz modulation frequency, at room temperature.

Determination of Förster Distances (R_0) for Fluorophore–Quencher Pairs. Corrected emission spectra of fluorophores were recorded at 1-nm wavelength resolution. Absorption spectra of quenchers were also recorded at 1-nm resolution in a Hewlett-Packard 8452A diode-array spectro-

photometer, at known concentration. The overlap integral J was calculated as follows:

$$J = \int_0^\infty F_D(\lambda)\epsilon_a(\lambda)\lambda^4\, d\lambda$$

The Förster distance (R_0) at which the efficiency of resonance energy transfer is 50% was then calculated according to

$$R_0^6 = (8.78 \times 10^{-5})\kappa^2 n^{-4}\, \Phi_D J \qquad (Å^6)$$

where κ^2 is a factor determined by relative orientation of donor and acceptor dipoles (a statistically averaged value of $^2/_3$ was used for κ^2), n is the refractive index of the medium surrounding the probes ($n \approx 1.4$), Φ_D is the quantum yield of donor luminescence, and J is the spectral overlap integral.

Results

Quenching of Luminescence from Tb^{3+} Chelates and Fluorescein by TEMPO Derivatives in Aqueous Solution. We have investigated the quenching of lumiescence by nitroxide radicals of singlet and triplet excited states of two different luminophores, in aqueous solution. The luminescent species were the Tb^{3+}/EDTA complex, Tb^{3+}/CITC-conjugated KE2 antibody, free fluorescein, and FITC-conjugated KE2 antibody. Two TEMPO derivatives, TEMPO-choline (positively charged) and 4-hydroxy-TEMPO (neutral) were used as water-soluble quenchers.

Tb^{3+} (2 mM) in Na^+,K^+-Hepes buffer was weakly fluorescent when excited at 295 nm. When EDTA or CITC–KE2 antibody conjugate was added, the characteristic emission peaks of complexed Tb^{3+} ions appeared at 488 and 545 nm accompanied with a significant enhancement of the quantum yield. In all quenching experiments, we investigated the emission from the long-lived $^5D_4$ state of Tb^{3+} (peak at 545 nm), which is assumed to be a triplet state. The intensity of the luminescence from Tb^{3+}/CITC–KE2 complex was about 1.5-fold higher than that of the Tb^{3+}/EDTA complex.

In order to examine the specificity of the enhancement of Tb^{3+} luminescence by CITC chelation, various concentrations of Tb^{3+} were added to solutions of CITC–KE2, unlabeled KE2, and ITC-EECP-labeled KE2 antibodies. The luminescence intensity of the 545-nm emission peak increased as Tb^{3+} concentration was raised with an abrupt increase of intensity around 1 mM Tb^{3+}. The intensity of the luminescence was significantly higher in the chelator–antibody (CITC–KE2) solution than in solutions of the two other antibodies (data not shown). Although there was some luminescence enhancement due to nonspecific interaction of Tb^{3+} with KE2 antibody, chelation by CITC results in a much larger enhancement. These results indicate that CITC enhances Tb^{3+} luminescence effectively, and in a 150 nM CITC-KE2 solution the luminescence intensity plateaus at around 1 mM Tb^{3+}.

Luminescence of the Tb^{3+}/EDTA or Tb^{3+}/CITC complexes was strongly quenched by both TEMPO-choline and TEMPOL, in a concentration-dependent fashion. No quenching was observed when the samples were pretreated with ascorbate (to reduce the spin label), suggesting that the nitroxide radical is essential for the quenching (Figure 1). A small but significant difference can be observed between the Stern–Volmer constants of TEMPOL and TEMPO-choline.

The spin labels also proved to be very efficient quenchers of fluorescein emission in aqueous solution, at neutral pH (Figure 1). Both free and antibody-conjugated fluorescein were strongly quenched, though the conjugation resulted in a small decrease in quenching efficiency (Table I). None of the fluorophores showed a spectral shift in emission upon addition of the quencher, up to 50 mM concentration.

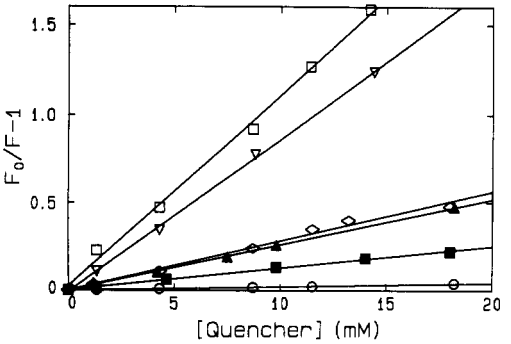

FIGURE 1: Stern–Volmer plot of quenching of Tb^{3+}/EDTA and fluorescein fluorescence by TEMPOL and TEMPO-choline water-soluble spin labels. The Tb^{3+}/EDTA complex was dissolved in 10 mM Hepes (145 mM NaCl, 5 mM KCl, pH 7.4) to a final concentration of 5 mM. The fluorescence intensity of the 545-nm emission peak was measured at various concentrations of TEMPOL (□) and TEMPO-choline (▽) at 20 °C. The quenching by TEMPOL of samples pretreated with 1 mM (◇) and 10 mM (○) ascorbate as well as quenching of free (▲) and antibody-bound fluorescein (■) by TEMPOL is also shown.

Table I: Stern–Volmer Constants and Apparent Quenching Rate Constants Determined for Different Fluorophore–Quencher Pairs in Neutral Aqueous Solutions[a]

emitter	quencher	K_{sv} (M^{-1})	k_q (M^{-1} s^{-1})
fluorescein	TEMPOL	26.8	6.7×10^9
fluorescein	iodide	7.2	1.8×10^9
Tb^{3+}/EDTA	TEMPOL	109.8	9.8×10^4
Tb^{3+}/EDTA	TEMPO-choline	90.7	8.1×10^4
Tb^{3+}/EDTA	iodide	0.09	$<1.0 \times 10^2$
sulfo-Rhodamine	TEMPOL	10.2	3.2×10^9

[a] The quenching rate constants were calculated from the slopes of Stern–Volmer plots using the experimentally determined lifetimes for the fluorophores (Table II, Figure 3a,b). For sulfo-Rhodamine a lifetime of 3.2 ns (Green et al., 1973) was used. All the rate constants were determined at 20 °C, in 10 mM Hepes buffer (pH 7.4).

For both luminophores, plotting the quenching data according to the Stern–Volmer equation

$$F_o/F = 1 + K_{sv}[Q]$$

resulted in linear functions up to 50 mM quencher concentration (Figure 1). Here K_{sv} is the quenching constant, which can be interpreted in different ways depending on the assumed mechanism of the quenching. For purely dynamic (collisional) quenching, it is defined as

$$K_{sv} = k_q\tau_o$$

where k_q is the quenching rate constant and τ_o is the excited-state lifetime in the absence of the quencher. k_q is proportional to the rate of the diffusion-controlled bimolecular collisional reaction, k_d, as

$$k_q = \sigma k_d$$

where σ is the efficiency factor of the quenching. In another limiting case, for static quenching, K_{sv} can be defined as an association constant for the complex of the ground-state fluorophore and the quencher. In this case, the quenching reaction is assumed to proceed instantaneously at the moment of excitation.

For fluorescein, the quenching rate constants calculated from the Stern–Volmer plot, using a lifetime of 4 ns (Table II), were relatively close to the diffusion-controlled limit (ca. 5×10^9 M^{-1} s^{-1} at 20 °C). In contrast, the apparent quenching rate for Tb^{3+} luminescence (using a lifetime of 1.12 ms) was about 4 orders of magnitude smaller (ca. 9.8×10^4 M^{-1} s^{-1})

Table II: Decay of Fluorescein Fluorescence in Free and Protein-Conjugated Form in Neutral Aqueous Solution: Effect of 4-Hydroxy-TEMPO Quencher on the Lifetime[a]

sample	lifetimes (τ_i) (ns)	amplitudes (α_i)
fluorescein	τ_1 0.63	α_1 0.12
Hepes, pH 7.4	τ_2 3.96	α_2 0.88
FITC-KE2 F_{ab}	τ_1 1.32	α_1 0.28
Hepes, pH 7.4	τ_2 4.11	α_2 0.72
FITC-KE2 F_{ab}	τ_1 1.06	α_1 0.28
+10 mM quencher	τ_2 3.79	α_2 0.72
FITC-KE2 F_{ab}	τ_1 0.95	α_1 0.28
+24 mM quencher	τ_2 3.36	α_2 0.72

[a] The lifetime and amplitude data were obtained from an analysis based on nonlinear least squares for double-exponential decay which gave the best fit ($\chi^2 < 1.5$) to the experimental data. The experimental data were taken by frequency-domain fluorometry from 1 to 200 MHz.

than the diffusion-controlled limit (Table I).

In order to interpret these data, we looked to see if the basic criteria for a diffusion-controlled collisional quenching are fulfilled by our system. According to these criteria, the quenching rate is expected to increase with increasing temperature and to decrease strongly with increasing viscosity. Also, the luminescence lifetimes are expected to decrease with increasing quencher concentration to a same extent as the steady-state intensities do. In contrast, a static quenching mechanism could be inferred from a lack of viscosity dependence, a decrease in quenching with increasing temperature, or an absence of change in excited-state lifetime upon addition of quencher. The quenching reaction was also examined for evidence of "long-range" energy-exchange mechanisms, such as Förster-type resonance energy transfer or electron transfer, which are also considered as potential relaxation pathways.

Effect of Quenchers on the Excited-State Lifetimes of Fluorescein and Tb^{3+}. The decays of fluorescein and Tb^{3+} luminescence were analyzed with frequency-domain and time-domain fluorometry, respectively. In Na^+,K^+-Hepes buffer at pH 7.4 and room temperature, the fluorescence decay curves of both free and antibody(KE2-F_{ab})-conjugated fluorescein were best fit to a double-exponential decay function. For fluorescein, the two lifetime components were 0.6 and 3.9 ns with fractional amplitudes of 0.12 and 0.88, respectively, in good agreement with earlier data (Lakowicz, 1983). Conjugation of fluorescein to the F_{ab} fragment of the KE2 antibody resulted in slightly longer lifetime components (1.3 and 4.1 ns, respectively) with slightly altered amplitude fractions (0.28 and 0.72, respectively). TEMPOL reduced both lifetime components, leaving the relative amplitudes unchanged (Table II). The extent of the lifetime reduction (i.e., the slope of the Stern-Volmer plot) was only slightly smaller (by about 13%) than the decrease of the corresponding steady-state fluorescence intensities.

For Tb^{3+}/EDTA in Na^+/K^+-Hepes buffer at pH 7.4 and room temperature, a single-exponential decay function with a lifetime of 1.12 ms gave an excellent fit to the experimental data. TEMPOL shortened this lifetime in a concentration-dependent manner (Figure 2). Again, the extent of lifetime reduction was nearly the same as that of the steady-state fluorescence intensity. Both time-resolved Stern-Volmer plots were linear over the entire range of quencher concentration (0-30 mM).

Temperature Dependence of the Quenching. Quenching of fluorescence by TEMPOL of KE2F_{ab}-conjugated fluorescein and Tb^{3+}/EDTA was analyzed between 6.0 and 42 °C. The extent of the quenching was practically independent of temperature in this interval for Tb^{3+}, and a slight decrease of the quenching rate with increasing temperature was observed for fluorescein (Figure 3). The linearity of the Stern-Volmer plots was unchanged within this temperature range at quencher concentrations of 0-40 mM.

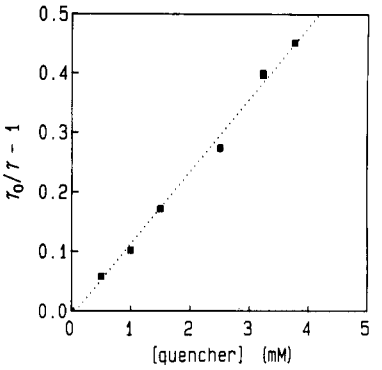

FIGURE 2: Time-resolved Stern-Volmer plot of the quenching of Tb^{3+}/EDTA fluorescence by TEMPOL in aqueous solution. The lifetimes were determined as described under Materials and Methods. (The unquenched lifetime was 1.12 ms, and all the decay curves showed a good fit to single-exponential functions.)

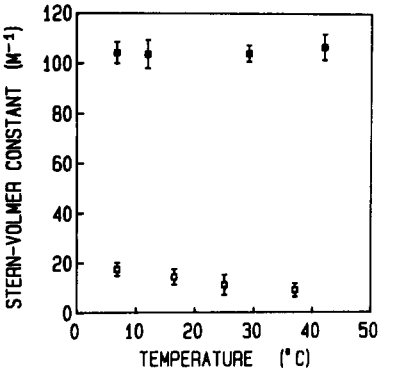

FIGURE 3: Temperature dependence of quenching by TEMPOL of Tb^{3+} and fluorescein luminescence. Fluorescein-conjugated KE2 F_{ab} (□) and Tb^{3+}/EDTA (■) were dissolved in 10 mM Hepes (145 mM NaCl, 5 mM KCl, pH 7.4) and then titrated with small aliquots of TEMPOL stock solution at the temperatures indicated.

Viscosity Dependence of the Quenching. Viscosity dependence of the quenching rates for both Tb^{3+}/EDTA and fluorescein-KE2F_{ab} was investigated in the viscosity range of 1-300 mPa·s, at 20 °C. The quenching constants showed an apparent independence of solution viscosity in the range of 1-60 mPa·s after a slight initial increase with increasing viscosity, in the low viscosity range (1-8 mPa·s). Only a moderate viscosity dependence could be observed even at extremely high (>80 mPa·s) viscosities (Figure 4). The same behavior (viscosity profile) obtained when using chemically different compounds to raise the viscosity of the solutions: the polyhydroxy compounds glycerol, sucrose, dextran, or (polyvinyl)pyrrolidone (PVP). The theoretically expected K_Q values calculated on the basis of the Smoluchowski and Einstein-Stokes equations (Lakowicz, 1983) for an ideal diffusion-controlled reaction are also displayed on Figure 4 for comparison, together with experimentally determined quenching constants for the quenching of fluorescein by a widely used collisional quencher, iodide. The quenching constants for iodide showed a good fit to the calculated function, indicating that this reaction, unlike the others, is really diffusion-controlled, as it was expected in the light of earlier findings.

Testing for Long-Range Energy-Exchange Mechanisms of Quenching. All the results shown previously, except the reduction in lifetime, suggest that the rate-limiting step of the

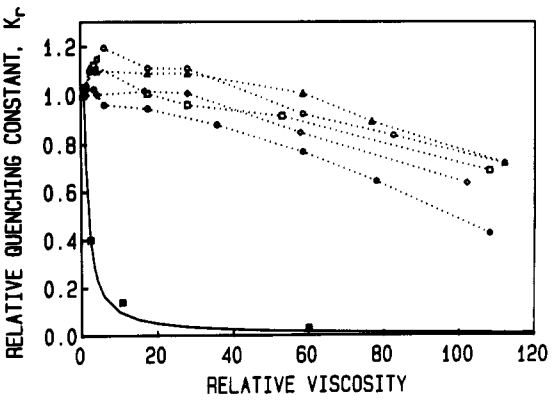

FIGURE 4: Viscosity dependence of the quenching of Tb^{3+}/EDTA and fluorescein fluorescence. The relative quenching constants were obtained from Stern–Volmer analysis of quenching data followed by a normalization to the value measured in aqueous solution (water viscosity) at 20 °C. These values are displayed vs relative viscosities calculated by normalization to the viscosity of water (1 mPa·s). The quencher was 4-hydroxy-TEMPO in all cases. The quenching constants for fluorescein in sucrose (○), PVP (◇), and glycerol (●) and for Tb^{3+}/EDTA in sucrose (▲) and glycerol (□) are shown as a function of relative viscosity (dotted lines). Quenching constants calculated for an ideal diffusion-controlled collisional quenching reaction are displayed as a solid line. Quenching constants for quenching of fluorescein fluorescence by iodide, determined experimentally at several relative viscosities (■), are also shown.

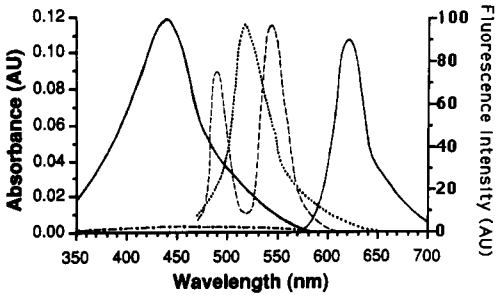

FIGURE 5: Emission spectra of Tb^{3+}/EDTA (dashed line), fluorescein (dotted line), and sulfo-Rhodamine (solid line, right) and the absorption spectrum of 4-hydroxy-TEMPO (solid line, left) are shown. The absorption spectrum of 4-hydroxy-TEMPO after treatment with 10 mM ascorbate (–··–) is also shown.

quenching reaction is not a diffusion-controlled collisional encounter and it is not static (ground-state complex) either. Therefore, we tested for evidence of other "through-space" quenching mechanisms such as Förster-type resonance energy transfer (dipole–dipole coupling) (Förster, 1949) and electron exchange (transfer) (Dexter, 1953; Marcus, 1956; Meares et al., 1981). A prerequisite of Förster-type energy transfer is an extensive spectral overlap between the emission spectrum of donor and the absorption band of acceptor (quencher). The TEMPO derivatives have a broad visible absorption band (peak at ca. 440 nm) showing a small but significant spectral overlap with emission spectra of both emitters (Tb^{3+}, fluorescein) (Figure 5). The low value of spectral overlap integral is mostly due to the low molar absorption coefficients of quenchers (see Materials and Methods). The R_0 values were calculated from spectral data and were found to be 1.14 and 1.0 nm for fluorescein/TEMPOL and Tb^{3+}/TEMPOL pairs, respectively. Though these low R_0 values make dipole–dipole coupling less probable, it still could be significant at shorter distances. It is noteworthy that reduction of the radicals by ascorbate, which abolished the quenching in concentration-dependent manner, also abolishes the visible absorption band of nitroxide quenchers, in this way eliminating the spectral overlap (Figures 1 and 5).

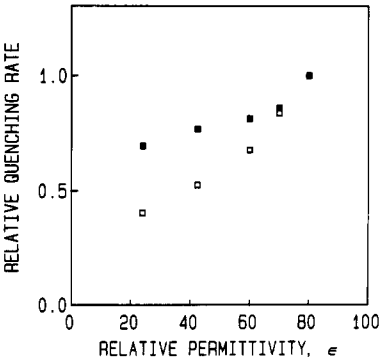

FIGURE 6: Effect of solvent properties on the quenching rate for quenching of Tb^{3+}/EDTA (■) and fluorescein (□) luminescence by TEMPOL. The relative permittivity (dielectric constant) of the solution was varied by changing the ratio (v/v) of the components in dioxane/water mixtures. (The temperature was 20 °C.) The quenching constant measured at 100% water/0% dioxane was taken as unit.

The fluorescence of sulfo-Rhodamine 101 (Texas Red) is red-shifted relative to the other two probes (emission peak at ca. 615 nm) and thus does not overlap with the quenchers' absorption band at all (Figure 5). However, this fluorescence could also be quenched by TEMPO quenchers, though to a smaller extent. The quenching constant K_{sv} (at 20 °C, pH 7.4, in aqueous solution) was 10.2 M^{-1}, compared to the values of 26.8 M^{-1} and 109.8 M^{-1} obtained with fluorescein and Tb^{3+}/EDTA, respectively. Furthermore, PROXYL derivatives as quenchers also quench all the three luminescences (data not shown) although their spectral overlap with the luminophores' emission spectra is practically zero due to the undetectably low absorbance in the visible spectral region. These findings together suggest a lack of correlation between the extent of quenching and the spectral overlap.

Another criterion of Förster- or electron-exchange(transfer)-type quenching interactions is the strong dependence on properties of solvent surrounding donor and acceptor molecules. As Figure 6 demonstrates, the quenching rate proved to be sensitive to the dielectric constant (relative permittivity) of the solution in water/dioxane mixtures. The changes in ϵ did not alter the shape of emission spectra significantly but resulted in shifts of excitation spectra and quantum yield. The sensitivity of the rate constants to ϵ were different for the singlet- and triplet-state probes, but both rates decreased with decreasing electrostatic dielectric permittivity. Ionic strength, as expected for electrically neutral species, did not significantly alter the quenching by TEMPOL but slightly influenced the rate for TEMPO-choline (data not shown).

The dependence of the quenching rate on the separation distance between fluorophores and nitroxide radicals was experimentally tested in a system which is very similar to that used by Stryer and Haugland (1967) to demonstrate the sixth power distance dependence of Förster-type resonance energy transfer. We measured the extent of quenching in a series of peptides having the following common structural formula: F–Thr–$(Pro)_n$–Q. The donor (F) was fluorescein while the "dark acceptor" (Q) was PROXYL (EECP) in these peptides. Using EECP moiety as acceptor practically eliminates the contribution from Förster-type energy transfer since PROXYLs unlike TEMPOs do not have detectable absorbance in the spectral region where fluorescein emits. Significant quenching (35–40%) was detected in the peptide with one proline (n = 1), and the quenching rate declined very sharply within a ca. 10-Å separation distance interval, so as no quenching could be detected in peptides with $n > 4$. The separation distances were taken from Stryer and Haugland

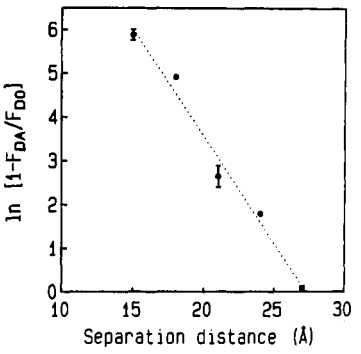

FIGURE 7: Distance dependence of the quenching of fluorescein fluorescence by PROXYL spin label in oligoproline peptides of different length. The quenching was measured as described under Materials and Methods. The logarithm of quenching probability is displayed as a function of separation distance between the covalently attached fluorophore and quenchers molecules. (The error bars represent typical standard deviations of quenching measurements.)

(1967), and an additional 3 Å was allowed for the contribution of the single threonine residue in each peptides. The distance dependence of quenching rates could be satisfactorily fit to an exponential function (Figure 7).

DISCUSSION

Since its suggestion by Jost et al. (1971) and introduction by Bieri and Wallach (1975), the technique of quenching of fluorescence by paramagnetic probes has been widely used for quantitative characterization of molecular interactions in both model and biological membranes [e.g., London and Feigenson (1978, 1981a,b), Atik et al. (1979), London (1982), Blatt and Sawyer (1985), Chattopadhyay and London (1987), and Yeager and Feigenson (1990)]. The analysis of the quenching data, however, faced the problem that the distance dependence of the quenching was unknown and there were conflicting results and conclusions about the mechanism (Buchachenko et al., 1967; Birks, 1970; Green et al., 1973; Gijzeman et al., 1973c; Kuzmin & Tatikolov, 1977; Atik & Singer, 1978; Puskin et al., 1981] due mostly to the complexity of the relaxation pathways (Green et al., 1990). Several authors (Green et al., 1973; Atik & Singer, 1978; London & Feigenson, 1981a) reported that the quenching interaction in solution is essentially dynamic (collisional) with an effective interaction range of 0.5–0.7 nm. In contrast, others argued that in many cases long-range resonance energy transfer interactions may have a significant contribution to the observed quenching (Buchachenko et al., 1967; Birks, 1970; Puskin et al., 1981). Very recently, Green et al. (1990) proposed an electron-exchange-type quenching mechanism for *intramolecular* quenching in some paramagnetic organic compounds. Due to the large diversity of experimental conditions and solvent compositions used in these earlier studies, the principal physical cause of the quenching still remained unclear, particularly in aqueous solutions.

In neutral aqueous solution, we found some characteristic similarities and differences between quenching reactions of the triplet-state Tb^{3+} and the singlet-state fluorescein with nitroxide quenchers. A striking difference is that while the fluorescein is quenched at a rate ($\approx 5 \times 10^9$ M^{-1} s^{-1}) close to the diffusion-controlled limit, the Tb^{3+} is quenched at a much slower rate ($\approx 9.8 \times 10^4$ M^{-1} s^{-1}). This low quenching rate for Tb^{3+} is inconsistent with a picture of diffusion-controlled collisional quenching if we consider that the excited-state lifetime of Tb^{3+} is about 6 orders of magnitude longer (1.12 ms) than that of fluorescein (4 ns). This might mean either that the two probes are quenched with entirely different mechanisms or that the quenching rate is not limited by the diffusional properties of the reactants.

The rates obtained for singlet probes (Table I) fall in the range of typical diffusional quenching rates reported for aromatic hydrocarbons and nitroxides (Green et al., 1973, 1990; Kuzmin & Tatikolov, 1977). The quenching rate for the triplet-state Tb^{3+} is similar to rates reported for phosphorescent tryptophans in a large number of different proteins in solution (Calhoun et al., 1988; Vanderkooi et al., 1990; Mersol et al., 1991). Slightly higher rates were found for the quenching of triplet states of aromatic hydrocarbons by radicals (Caldwell & Schwerzel, 1972; Gijzeman et al., 1973a,b; Gijezeman & Kaufman, 1973; Kuzmin et al., 1978), and a correlation between triplet-state energy levels and the quenching rate was postulated. Higher energy triplets were predicted to be quenched at more rapid rates, and indeed some experimental data [e.g., quenching of retinal triplet state or eosin derivatives by nitroxide radicals with rate constants of 10^8–10^9 M^{-1} s^{-1} (Kuzmin et al., 1980; Koloczek & Vanderkooi, 1987)] seem to support this hypothesis.

Most of the earlier work postulated that, regardless of the actual mechanism, quenching of excited singlet or triplet states by nitroxide radicals occurs at the diffusion limit (Green et al., 1973; Chattopadhyay et al., 1983; Closs et al., 1986). Closs et al. (1986), pointing out that efficient electron transfer is not restricted to systems where the donor and the acceptor are in physical contact or close proximity, argued that in intermolecular electron transfer processes the diffusion completely masks kinetic properties of the electron transfer (e.g., solvent dependence). In contrast, both our model systems showed a characteristic temperature and viscosity dependence which argue strongly against a diffusion-controlled quenching mechanism. In addition, we observed a relatively strong solvent dependence for quenching of both probes. The lack of significant temperature and viscosity effects on the quenching rate, in a relatively broad scale of these variables, both indicate that the quenching rate is apparently independent of the diffusional properties of the reactants even in isotropic solution. Kinetically, it may reflect involvement of a slower rate-limiting step in the quenching reaction.

Only a very few examples are known from the literature that show kinetic behavior similar to that described here. Vanderkooi and co-workers observed an apparent viscosity independence in the quenching of the triplet excited state of a variety of protein tryptophans by added small molecules (Calhoun et al., 1988; Vanderkooi & Berger, 1989; Vanderkooi et al., 1990). Similarly a "viscosity-independent part" was observed in the quenching of synthetic fluorescent "membrane-spanning peptides" by brominated phospholipids in unilamellar vesicles (Bolen & Holloway, 1990). In contrast, the quenching of another triplet-state probe, eosin, by TEMPOL proved to depend on solution viscosity (Koloczek & Vanderkooi, 1987).

The sensitivity of the observed quenching rate to solution properties (temperature, viscosity) seems to correlate with the energy gap between the excited-state energy levels of the fluorophores and the quencher and this way with the magnitude of the rate constants. In other words, the energetic conditions for quenching (e.g., degree of exothermicity) may define kinetic behavior of the quenching reaction (Turro, 1978).

Our experimental data allow us to suggest a generalized conclusion about kinetic features of the quenching reaction between singlet and triplet excited states and radical quenchers.

In solution, the observed quenching rate depends on the relationship between the diffusion rate and the rate of the process in which the reactants exchange the excitation energy. It is reasonable to assume that the small probe molecules or the labeled antibody fragments diffuse freely in aqueous solution and do not react chemically with each other. Thus, the above statement, in full accordance with a recent proposal of Vanderkooi and Berger (1989), can be formulated by the kinetic scheme

$$L^* + Q \xrightarrow{k_d} (L^*Q) \xrightarrow{k_{EE}} (LQ^*) \xrightarrow{k_{-d}} L + Q^*$$

where k_d is the rate of collisional encounter, k_{-d} is the breakup rate of the collisional complex, and k_{EE} is the rate of the exchange of excitation energy between an excited luminophore and a quencher molecule. If the exchange rate, k_{EE}, is much slower than k_d, the consequence is an apparent independence of the observed quenching rate, k_q, of the diffusional properties of reactants. At extremely high viscosities, the two rates may become comparable or the transfer rate can even exceed the k_d, resulting in viscosity dependence of k_q as it was observed in our case at viscosities >80 mPa.s. This model is also consistent with our finding that the excited-state lifetime was reduced with increasing quencher concentration.

The exchange rate itself may depend on several factors, among others, the electronic structure (energy levels) and chemical properties of both the emitters and the quenchers, redox properties of the system, and the local solvent properties in the volume elements around and between the reactants (Robinson & Frosch, 1962; Hoytink, 1969; Gijzeman et al., 1973a,b; Gijzeman & Kaufman, 1973). These features of the electron exchange may give an explanation of the kinetically surprising viscosity independence of the quenching rate for fluorescein in a relatively broad viscosity range. We think that the viscosity profile for quenching of fluorescein reflects a composite of at least two effects. Increasing viscosity slows the diffusion of the reactants. On the other hand, the increasing concentration of viscosity-elevating compounds can modify the energetic conditions for electron exchange (Turro, 1978) through "solvent effects" on the electronic energy levels of both fluorescein and the nitroxide spin label (Mukerjee et al., 1982). Indeed, we observed spectral shifts with fluorescein as a function of glycerol or sucrose concentration in contrast to the practically unchanged spectra of Tb^{3+}. An efficient quenching by electron exchange with high sensitivity to the degree of exothermicity was reported by Miller et al. (1982) in rigid solutions. A contribution of triplet states formed by quencher-enhanced intersystem crossing (Kuzmin & Tatikolov, 1977) or some incomplete static quenching by partial charge transfer may further complicate the picture about quenching of fluorescein by TEMPOL.

The quenching of singlet and triplet excited states by nitroxides could be due to several of a number of exchange mechanisms. Thus, the observed quenching rate constant can be written as

$$k_q = k_{FT} + k_{ET} + k_{EE} + k_{CT} + k_S$$

that is, as a sum of the potential deexcitation pathways, which are the Förster-type resonance energy transfer (FT) (Buchachenko et al., 1967; Puskin et al., 1981), electron transfer (ET) (Marcus, 1956; Mayo et al., 1986), electron exchange (EE) (Dexter, 1953; Hoytink, 1969), partial or complete charge transfer (CT) (Kuzmin et al., 1978), and a static ground-state complex formation (S), respectively.

Formation of dark ground-state complexes ("classical static quenching") has a low probability in solution at low concentrations and can be ruled out since in that case no change in the excited-state lifetime is expected, while spectral shifts are often observed (Lakowicz, 1983). Our experimental findings are inconsistent with this mechanism, at least in the millimolar concentration range of the quenchers.

Förster-type resonance energy transfer also does not make a significant contribution to quenching, in contrast to proposals by Buchachenko et al. (1967) or Puskin et al. (1981). We found a small but nonnegligible spectral overlap between emission spectra of both the triplet and singlet probes and absorbance of nitroxide quenchers. However, a complete lack of correlation between the extent of spectral overlap and the quenching rate was demonstrated with the use of sulfo-Rhodamine 101 fluorophore or the PROXYL quenchers. These observations are in good agreement with the finding of Koloczek and Vanderkooi (1987) that eosin is quenched by TEMPOL with a high rate constant in spite of lack of spectral overlap. Very recent, detailed investigations of Green et al. (1990), in conformity with our results, convicingly demonstrate that Förster-type transfer cannot play significant role in the quenching by spin labels.

The most likely mechanism of the quenching in our experiment is an electron exchange (transfer). For triplet probes, it occurs most often as a quencher-catalyzed intersystem crossing in the luminophore molecule following a collision between the reactants. Singlet excited molecules can also be deactivated by exchange interactions in the collisional complex with a rate predicted to be close to the diffusion-controlled limit (Hoytink, 1969), also in accordance with our findings with fluorescein and fluoresceinated antibody. In the latter case, the lower rate constant is probably due to some restrictions to the collisions of the quencher molecules with the bound fluorophores by the surrounding protein matrix or to direct effects of protein moieties on the exchange rate.

According to a detailed theoretical and experimental analysis by Robinson and Frosch (1962), Gijzeman & Kaufman (1973), and Gijzeman et al. (1973a,b), a partial charge transfer state may also contribute to the quenching depending on the ionization potential of donor, the electron affinity of quencher, and the relationship between the energy levels of the triplet and charge transfer states. They predicted that an increase in the solvent polarity may result in a rate enhancement changing the relative contribution of charge transfer states and the exchange to the quenching (Gijzeman et al., 1973a; Figure 4). Our experimental results on solvent dependence are in excellent agreement with these predictions. The difference in the extent of solvent effects for the two probes is very likly due to a different probability for contribution of CT states in the quenching.

The rate constant for quenching by electron exchange was formulated by Dexter (1953) and Hoytink (1969) as

$$k_{ex}(r) = k_o \exp(-2r/L)$$

where k_o is the quenching rate constant at the van der Waals contact distance between donor andacceptor molecules, r is the actual separation distance between them, and L is an effective Bohr radius (typically 1 Å). It is noteworthy that quenching rates by electron transfer are formulated by the same function (Marcus & Sutin, 1985; Mayo et al., 1986) with a small difference that the value of L (effective distance for electron transfer) is typically ranging from 0.5 to 2.0 Å and very sensitive to the medium separating the donor and acceptor as well as to the redox state of the reactants, in good agreement with our ascorbate effects.

On the basis of X-ray crystallographic estimations of distances of Trp residues from the protein surface, Vanderkooi

et al. (1990) found a good fit to an exponential distance dependence of triplet quenching rate constants. We used a more direct way of calibration exploiting the nearly rigid nature of synthetic proline oligopeptides (Stryer & Haugland, 1967). The experimentally observed distance dependence of the quenching rate shows an excellent agreement with the exponential dependence predicted by the theories. These results also demonstrate that electron exchange/transfer interactions are not restricted to the case of physical contact; the effective interaction distances can be as long as 1.5–2 nm.

In the light of our findings, we can conclude that in aqueous solution intermolecular quenching of both singlet and triplet excited states by TEMPO and PROXYL-type nitroxide radicals occurs via an electron-exchange(transfer) mechanism. Using these quenchers, the contribution from Förster-type resonance energy transfer can be excluded, in contrast to brominated phospholipid quenchers (Bolen & Holloway, 1990). We also provided direct experimental evidence for exponential distance dependence of the quenching rate using a series of synthetic peptides.

These luminophore–quencher pairs are potentially useful probes to experimentally determine distances of triplet Trp residues from protein surface, although modeling of this problem, in spite of the recent progress (Vanderkooi et al., 1990, 1991), still needs further theoretical analysis. Another promising application of this "electron-exchange quenching" (EEQ) is to examine spatial proximity/physical association of cell surface membrane proteins with one another. Exploiting the extremely high sensitivity of the quenching probability to the separation distance between 0.8 and 2.0 nm, we expect that the quenching signal will be able to distinguish between physical association and the ill-defined "close proximity" inferred mostly from Förster-type resonance energy transfer measurements (Matko et al., 1988). EEQ obviously had the drawback that experimental determination of the "true quenching constant" is less accurate than in case of FRET since due to the dark acceptors there is no chance to measure acceptor sensitization. On the other hand, the approach has several obvious advantages, too. Since the effective distance interval of the quenching falls in the range of molecular radius of antigen or antibody molecules, a positive quenching signal will report, with high probability, a physical association between the labeled membrane proteins. Application of small size spin labels covalently attachable to antibodies gives further advantages of efficient quenching of a wide variety of luminophores and minimal perturbation of native antibody conformation, which is important to retain binding affinity to the cell surface antigens. In our preliminary investigations on living cells, we were able to detect association/clustering of some cell surface antigens while no quenching was observed in cases when noninteracting cell surface proteins were labeled (Matko et al., 1991). A detailed study of the application of these probes and EEQ to living cells will be presented in a subsequent paper.

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Determination of the Excited-State Lifetimes of the Tryptophan Residues in Barnase, via Multifrequency Phase Fluorometry of Tryptophan Mutants†

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ABSTRACT: A multifrequency phase fluorometric study is described for wild-type barnase and engineered mutant proteins in which tryptophan residues have been replaced by less fluorescent residues which do not interfere with the determination of the tryptophan emission spectra and lifetimes. The lifetimes of the three tryptophans in the wild-type protein have been resolved. Trp-35 has a single fluorescence lifetime, which varies in the different proteins between 4.3 and 4.8 ns and is pH-independent between pH 5.8 and 8.9. Trp-71 and Trp-94 behave as an energy-transfer couple with both forward and reverse energy transfer. The couple shows two fluorescence lifetimes: 2.42 (±0.2) and 0.74 (±0.1) ns at pH 8.9, and 0.89 (±0.05) and 0.65 (±0.05) ns at pH 5.8. In the mutant Trp-94 → Phe the lifetime of Trp-71 is 4.73 (±0.008) ns at high pH and 4.70 (±0.004) ns at low pH. In the mutant Trp-71 → Tyr, the lifetime of Trp-94 is 1.57 (±0.03) ns at high pH and 0.82 (±0.025) ns at low pH. From these lifetimes, one-way energy-transfer efficiencies can be calculated according to Porter [Porter, G. B. (1972) *Theor. Chim. Acta 24*, 265-270]. At pH 8.9, a 71% efficiency was found for forward transfer (from Trp-71 to Trp-94) and 36% for reverse transfer. At pH 5.8 the transfer efficiency was 86% for forward and 4% for reverse transfer (all ±2%). These transfer efficiencies correspond fairly well with the ones calculated according to the theory of Förster [Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55-75]. The fluorescence lifetime of Trp-94, as determined in a mutant which lacks Trp-71, is heavily quenched by the neighboring imidazole group of His-18.

Fluorescence spectroscopy has been used extensively in the study of the time dependence of conformational changes in proteins. An interpretation of these fluorescence changes at the molecular level is possible only when they can be correlated with the environment of the fluorescent probe which is used. The fluorescence of proteins is usually dominated by the contribution of tryptophan residues. A correlation of the tryptophan environment to the fluorescence properties of the protein is possible when the structure of the protein is known (Longworth, 1983) and there is just one tryptophan residue. The same is true for proteins containing several tryptophan residues if the fluorescence lifetimes of the individual residues are resolved. In the present study, the fluorescence properties of barnase, an extracellular ribonuclease from *Bacillus amyloliquefaciens*, were determined by multifrequency phase fluorescence spectroscopy. The method relies on excitation

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