

Diffusion Coefficients of Quenchers in Proteins from Transient Effects in the Intensity Decays*

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We used 2-GHz frequency-domain fluorometry to examine the intensity decays of *N*-acetyl-L-tryptophamide (NATA) and the protein staphylococcal nuclease in the presence and absence of quenching by oxygen or acrylamide. When analyzed with a multiexponential model, the decays of NATA and nuclease both become more heterogeneous in the presence of quenching. We attribute the increased complexity to transient effects in quenching or equivalently a time-dependent rate constant for quenching. The frequency-domain data were analyzed using the Smoluchowski model ($\exp(-t/\tau - 2b\sqrt{t})$) and the radiation model, which is known to correct some flaws in the more approximate Smoluchowski model. The radiation model provides improved fits to the data, as evidenced by average 10-fold decreases in χ_r^2 . The radiation model also provides an estimate of the sum of the diffusion coefficients and the specific rate constant for quenching. The apparent diffusion coefficients for acrylamide and oxygen in nuclease, as seen by its single tryptophan (residue 140) are 15- and 11-fold lower than in water, respectively. The apparent values of the oxygen diffusion coefficient in water, as seen by NATA, are 2- to 3-fold larger than expected from earlier steady-state measurements. The ability to recover the detailed form of the intensity decays by the frequency-domain method should allow comparison of experimental results with calculated trajectories of quenchers in proteins.

Collisional quenching of fluorescence is of interest in chemistry and biochemistry. Quenching depends on diffusion-dependent encounters between fluorophores and the quenchers. Consequently, quenching has been used to study the diffusion of quenchers in proteins and membranes (1-7) and the accessibility of the membrane-bound fluorophores to quenchers (8, 9). To date, most researchers have used steady-state measurements of quenching, or used the decrease in the mean decay

time of the emission. However, the intensity decays contain additional information in the form of time-dependent rate constants, which are referred to as transient effects (10-13).

In this preliminary report we demonstrate that transient effects can be observed for oxygen and acrylamide quenching of the uncharged tryptophan derivative NATA¹ in water and for quenching of the single tryptophan residue (140) in staphylococcal nuclease. These effects were detected in the frequency domain (14, 15), using the harmonic content of a pulsed laser (16). The data were analyzed in terms of the Smoluchowski model (\sqrt{t}) and radiation boundary condition (RBC) model (17-19),² which yields estimates for the diffusion coefficient of the quencher and for the specific rate constant for quenching (κ).

THEORY

The theory for transients in quenching is too complex to present in detail in this preliminary report. Briefly, there are two practical models. The Smoluchowski model assumes that the fluorophore is quenched instantaneously when the quencher reaches a distance R from the fluorophore. Under this assumption the intensity decay is given by

$$I(t) = I_0(t) \exp\left(-\frac{t}{\tau} - 2b\sqrt{t}\right) \quad (1)$$

where

$$\frac{1}{\tau} = \frac{1}{\tau_0} + 4\pi RDN' \quad (2)$$

and

$$b = 4R^2N'\sqrt{\pi D} [Q]. \quad (3)$$

In this expression τ_0 is the decay time in the absence of quenching, R is the interaction radius, D is the sum of the diffusion coefficients of the fluorophore and quencher, N' is 6.02×10^{20} , and $[Q]$ is the molar quencher concentration. A second and probably more complete model is the RBC model (17, 18).² In this case one assumes that the fluorophore is quenched with a rate constant κ when at a distance R from the quencher. Then, the time-dependent rate constant for quenching is given by

$$k(t) = \frac{4\pi RDN'}{1 + D/\kappa R} \left[1 + \frac{\kappa R}{D} \exp(x^2) \operatorname{erfc}(x) \right] \quad (4)$$

where

$$x = \frac{\sqrt{Dt}}{R} \left[1 + \frac{\kappa R}{D} \right]. \quad (5)$$

The new parameter κ is a specific rate constant for quenching and has units of cm/s. The specific rate constant can be converted to the more familiar units of a bimolecular quenching constant ($M^{-1} s^{-1}$) by forming product $4\pi R^2N'\kappa$. The intensity decay is given by

$$I(t) = I_0 \exp\left(-\frac{t}{\tau_0}\right) \exp\left[-[Q] \int_{t=0}^t k(t) dt\right]. \quad (6)$$

We have already determined that the models are distinguishable when the extent of quenching is greater than 3- to 4-fold (19, 20). This is not surprising because the \sqrt{t} model is known to contain inconsistencies which are corrected in the RBC model (17, 18). Frequency-dependent values of the phase and modulation were obtained by numerical sine and cosine transforms of Equations 1 and 6 (19, 20). The values of D and κ are obtained from nonlinear least squares.

MATERIALS AND METHODS

Staphylococcal nuclease was a generous gift from Prof. L. Brand (The Johns Hopkins University). The sample was at least 98% pure

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¹ The abbreviations used are: NATA, *N*-acetyl-L-tryptophanamide; RBC, radiation boundary condition; \sqrt{t} , Smoluchowski model.

² Ref. 18 corrects an error in Ref. 17.

by high pressure liquid chromatography. Oxygen quenching was performed as described previously (3, 4, 21, 22). The frequency-domain data were measured using a 2-GHz harmonic-content fluorometer. The excitation wavelength was 298 nm from the frequency-doubled output of a R6G dye laser (16). Magic angle polarizer orientations were used to eliminate the effects of rotational diffusion. The emission was observed through a Schott WG320 bandpass filter, which absorbs all the emission and/or scattered light below 320 nm. For measurements in the oxygen pressure cell we used scattered light as the phase reference. For nuclease we used the intrinsic scatter; for NATA we added a trace amount of colloidal silica. Oxygen concentrations were calculated from the gas pressure, using the interpolated solubility at 20 °C (3, 4).

RESULTS

Fig. 1 shows the frequency response of the intensity decay of NATA. In the absence of oxygen the intensity decay is dominantly a single exponential, as can be seen from the overlap of the data (●) and the best single exponential fit (—). The deviations are randomly distributed (*lower panel*) and the goodness-of-fit parameter χ_R^2 is near unity (Table I), which indicate the data are adequately fit by the single exponential model (23). We have noticed that the intensity decay of NATA is weakly a double exponential (24), but this small degree of heterogeneity is not important for the present analysis. Quenching by oxygen (1500 p.s.i. at 20 °C, 0.1443 M) results in a shift in the frequency response to higher frequencies, which indicates a decrease in the mean decay time. Importantly, the data can no longer be described by the single exponential model, as is seen from the lack of agreement with this model (Fig. 1, —, *right*), the nonrandom deviations (*lower panels*) and the elevated value of $\chi_R^2 = 175$. The decay of NATA also becomes more complex in the presence of quenching by acrylamide, resulting in $\chi_R^2 = 85$ (Table I). Similar results have been reported for acrylamide quenching of indole (25).

We attribute the heterogeneous decay of quenched NATA to transient effects in quenching. These effects are due to the rapid extinction of closely spaced fluorophore-quencher pairs at early times following excitation, followed by slower diffu-

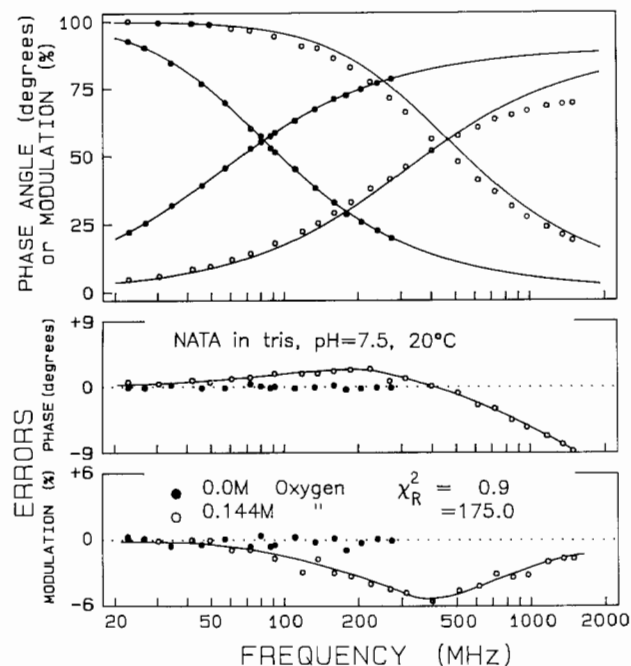


FIG. 1. Frequency response of NATA in the absence of oxygen (●) and when quenched by 0.144 M oxygen (○). The solid lines show the best single exponential fits to the data. The lower panels show the deviations between the data and the best single exponential fit.

TABLE I
Multiexponential analysis of NATA and staphylococcal nuclease in the presence and absence of quenchers

Sample	Quencher	τ_i	α_i	f_i	χ_R^2
NATA ^a	None	2.87	1.0	1.0	0.8
	0.1 M acrylamide	1.00	1.0	1.0	84.5
		0.28	0.25	0.07	
NATA	None	1.16	0.75	0.93	1.8
	0.144 M O ₂ (1500 p.s.i. O ₂)	2.95	1.0	1.0	1.0
		0.51	1.0	1.0	175
		0.05	0.37	0.05	
		0.59	0.63	0.95	1.6
Staphylococcal nuclease ^b	None	5.46	1.0	1.0	8.0
		4.60	0.68	0.57	
		7.31	0.32	0.43	1.2
	0.19 M acrylamide	3.41	1.0	1.0	17.3
		1.26	0.14	0.05	
		3.70	0.86	0.95	1.8
	None	5.42	1.0	1.0	5.9
		4.89	0.84	0.76	
		8.34	0.16	0.24	1.1
		0.143 (1485 p.s.i. O ₂)	1.70	1.0	1.0
0.29			0.36	0.07	
2.08			0.64	0.93	1.6

^a 25 mM Tris, pH 7.5.

^b 25 mM Tris, 100 mM NaCl, pH 7, 20 °C.

sion-limited quenching at longer times. If transient effects were not present, then the intensity decays of the quenched samples are expected to remain a single exponential. In fact, the widely used proof of collisional quenching, which is the equivalent decrease in the relative yields (F/F_0) and lifetimes (τ/τ_0), is based on the assumption that transient effects do not significantly alter the measured values. It is possible to fit the heterogeneity of the quenched decays by a multiexponential model (Table I). However, this model is not presently formulated in terms of the molecular features of the system. Consequently, we analyzed the data in terms of the radiation model (Equation 4), which has R , D , and κ as parameters, and the simple Smoluchowski model (Equation 1), which uses only R and D (Table II). In these analyses we generally held the encounter distance fixed because there is no reason to expect this value to vary and because the values of R and D are somewhat correlated. The encounter radii were assumed to be 7.0 and 5.5 Å, for acrylamide and oxygen quenching, respectively. In some cases we allowed both R and D to be variable parameters for the \sqrt{t} analysis. The data for oxygen-quenched NATA could not be fit using the Smoluchowski model. If R is held fixed at 5.5 Å, the failure is easily visible (Fig. 2, ---), and $\chi_R^2 = 61.5$. Our ability to detect deviations from the Smoluchowski model indicates the high resolution obtainable using the frequency-domain measurements. Better fits and lower values of χ_R^2 are found using the Smoluchowski model with both R and D variable (Table II). However, examination of several fluorophores and quenchers over a range of quencher concentrations revealed that the values of R and D become unrealistic at high quencher concentrations (19, 20). That is, we have independent evidence to suggest that the RBC model provides more realistic estimates of R and D than does the \sqrt{t} model.

The data for quenched NATA are better described by the RBC model (Table II). With R fixed at 5.5 Å we obtained an effective diffusion coefficient of 6.2×10^{-5} cm²/s and $\kappa = 505$ cm/s, with χ_R^2 decreased 9-fold to 7.0. The value of κ can be transformed to the more familiar units of M⁻¹ cm⁻¹ by forming $k = 4\pi R^2 N' \kappa$, which yields 1.16×10^{10} M⁻¹ s⁻¹. This indicates that the specific rate for quenching of NATA is approximately equal to the diffusion-limited rate, so that quenching by oxygen is diffusion limited. The diffusion coefficient is larger

TABLE II
Apparent diffusion coefficients and rate constants for quenching by oxygen and acrylamide

RBC model ^a	Quencher	$D \times 10^6$ <i>cm²/s</i>	κ <i>cm/s</i>	χ_R^2
NATA	0.1 M acrylamide	2.1	224	2.6
NATA	0.144 M oxygen	6.2	505	7.0
Staphylococcal nuclease	0.19 M acrylamide	0.14	32	1.3
Staphylococcal nuclease	0.143 M oxygen	0.58	242	4.3
\sqrt{t} model ^b		$D \times 10^6$ <i>cm²/s</i>	R \AA	χ_R^2
NATA	0.1 M acrylamide	0.79	(7.0)	8.9
NATA	0.1 M acrylamide	0.48	9.3	3.0
NATA	0.144 M oxygen	2.0	(5.5)	61.5
NATA	0.144 M oxygen	0.66	10.3	17.5
Staphylococcal nuclease	0.19 M acrylamide	0.05	(7.0)	8.0
Staphylococcal nuclease	0.19 M acrylamide	0.12	4.1	1.4
Staphylococcal nuclease	0.143 M oxygen	0.47	(5.5)	117.6
Staphylococcal nuclease	0.143 M oxygen	0.062	14.7	19.4

^a For these analyses the interaction radii were held constant at 7 \AA for acrylamide quenching and 5.5 \AA for oxygen quenching.

^b For these analyses the radius was either a variable parameter or held fixed at the values indicated in angle brackets ($\langle \rangle$).

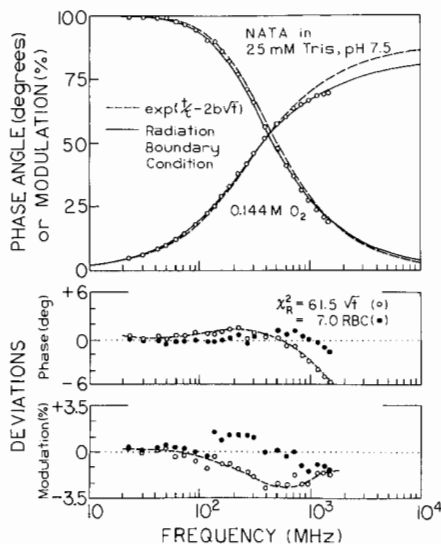


FIG. 2. Transient analysis of the frequency response of NATA with 0.144 M oxygen. The dashed line shows the best fit to the data using the Smoluchowski model, and the solid line shows the best fit using the RBC model. The lower panels are the deviations from the \sqrt{t} model (O) and from the RBC model (●).

than the expected value of $2.5 \times 10^{-5} \text{ cm}^2/\text{s}$ (3, 4). At present we do not know the cause of this discrepancy. In the present analysis the apparent value of D is determined by both the decrease in the mean decay time and by the transient effects, which probably reflect diffusion over short distances. The earlier estimates of D were obtained from either steady-state measurements or from the mean decay times. It is probable that these measurements did not contain information on the transient effects and may reflect primarily diffusion over longer distances. The present data may reflect the short-time transient effects due to mutual diffusion coefficients of NATA and oxygen over a short distance where intervening solvent molecules are less plentiful, as well as by the decrease in the mean decay time.

Similar data were obtained for the intrinsic tryptophan emission from nuclease. The emission from nuclease is due to a single tryptophan residue (140) near the end of the polypeptide chain, which extends to residue 149. This residue is near the outer surface of the protein and is found in a pocket formed by a peptide loop (26). This region of the structure is poorly defined, suggestive of conformational variability in the native structure. In the absence of quenching, the decay is close to a single exponential (Fig. 3). Actually, the decay is clearly a double exponential ($\chi_R^2 = 5.9$), but this heterogeneity is not presently important. Quenching by oxygen results in an increase in heterogeneity, as is seen from the increase in χ_R^2 from 6 to 265 (Table I). These data were analyzed using the two transient models (Fig. 4). Once again, the simpler

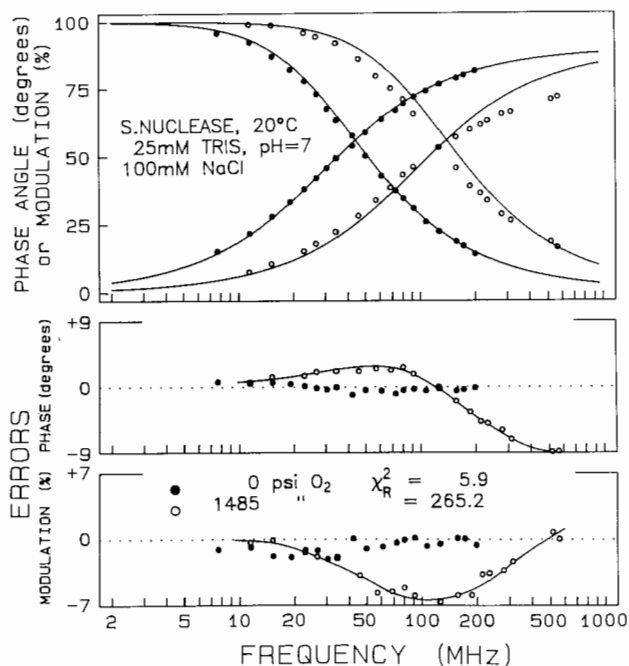


FIG. 3. Frequency response of staphylococcal nuclease in the absence (●) and presence (○) of 0.143 M oxygen. The solid lines show the best single exponential fits to the data. The lower panels show the deviations from the single exponential fit. *S. nuclease*, staphylococcal nuclease.

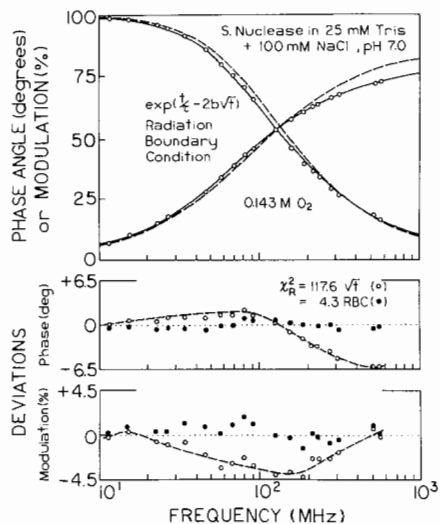


FIG. 4. Transient analysis of the frequency response of the emission from oxygen-quenched nuclease. The dashed line shows the best fit obtained using the \sqrt{t} model, and the solid line the best fit using the RBC model. *S. nuclease*, staphylococcal nuclease.

Smoluchowski model could not explain the data ($\chi_R^2 = 117.5$), whereas the RBC model provided an improved fit ($\chi_R^2 = 4.3$). The effective oxygen diffusion coefficient appears to be 11-fold less than for oxygen in water, as seen by NATA. Part of this decrease may be due to slower diffusion of the protein, so that the effect of shielding is probably closer to 9-fold. The value of κ is also decreased, from 505 for NATA to 242 cm/s for nuclease. At present, we do not have adequate experience to interpret the value of κ without ambiguity. It seems possible that the decrease in κ reflects steric barriers to quenching and/or a limited directional access of oxygen to the tryptophan residue.

Similar data were collected for acrylamide quenching of NATA and of nuclease (data not shown). Quenching of NATA by acrylamide resulted in increased heterogeneity in its intensity decay (Table I). Once again, the data appear to be better fit using the RBC model than the \sqrt{t} model (Table II). This distinction becomes greater as the quencher concentration is increased (not shown). A similar result was found for nuclease, as its decay becomes more heterogeneous in the presence of acrylamide quenching (Table I). The diffusion coefficient of acrylamide appears to be decreased 15-fold by nuclease, relative to that found for NATA in water. This is larger than was found for oxygen and may reflect a greater steric barrier for the larger acrylamide molecule. The κ value for acrylamide is also decreased more by nuclease than was the κ value for oxygen. This decrease in κ may reflect a limited angle of contact between the tryptophan residue and acrylamide or restrictions in the geometry of the contact. Additional experimentation is needed to interpret the values of κ and D recovered from the transient analysis.

And finally, the RBC model appears to provide a more consistent estimate of R and D . This is seen by comparing the fits from the \sqrt{t} model when R is held fixed or is allowed to vary (Table II). With R as a variable parameter one finds that acrylamide diffused more rapidly than oxygen within nuclease, and that the collision radius for tryptophan-oxygen is near 15 Å. From earlier studies we know that the approximation contained in the \sqrt{t} model results in unrealistic values of R and D , especially when data are obtained over a range of quencher concentrations (19, 20). While we now have evidence that the RBC model is not adequate for all cases,³ we believe it is the model of choice until a better theory can be developed.

DISCUSSION

The data and analyses demonstrate that the gigahertz frequency-domain measurements provide good resolution of the transient effects in quenching, even for small quenchers in media of low viscosity. The present analysis must be regarded with caution because we used the RBC model, which was derived for diffusion in homogeneous solution. We assumed this model also applies to a tryptophan residue in a protein. This assumption was necessary because we do not know of a usable theory for transient effects in the time-dependent decays of quenched fluorophores in two or more environments. Even if such a theory were available, it is likely that the number of parameters would be too large to recover from the data. Nonetheless, the RBC model yields apparent values for D and κ . We believe the values can be compared with other experimental or theoretical results. For instance, the

decays can be compared with calculated trajectories of quenchers in proteins, as has been performed for oxygen in hemoglobin (27). For this reason we summarized the results of the multiexponential analyses in Table I. These values may be regarded as a parameterized form of the intensity decays in the presence of quenching.

Our detection of transient effects in quenching have implications for the use of quenching to resolve classes of fluorescent residues in proteins (28, 29). The usual procedure is to obtain the wavelength-dependent decays and to assign the decay times to classes of residues. These decays are then measured at various concentrations of quenchers. Changes in the decay times are interpreted in terms of accessibility of each class to the quencher. However, our results demonstrate that quenching itself results in new components in the decay, even for an initially homogeneous fluorophore. Such effects may alter or invalidate the initial assignments even if they were correct. In fact, such effects have probably been observed by Weber and co-workers (30, 31) and Cheung *et al.* (32), but these were attributed to other causes.

It should be noted that one cannot construct a Stern-Volmer plot based on the decay times. Depending on the resolution of the experiment, one will obtain different values for the best single exponential fit. In our case the short components contribute a good deal to the data. If we construct a Stern-Volmer plot based on these values, then positive deviations are seen (see also Ref. 20). However, these are the result of using a single exponential approximation to a more complex decay, so the positive deviations are an artifact of the analysis and not a fundamental property of the system.

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³ J. R. Lakowicz, N. B. Joshi, M. L. Johnson, H. Szmanski, and I. Gryczynski, unpublished observations.