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VISCOSITY DEPENDENCE OF THE SOLUTE QUENCHING OF THE TRYPTOPHANYL FLUORESCENCE OF PROTEINS

Maurice R. EFTINK and Karen A. HAGAMAN *

Department of Chemistry, The University of Mississippi, University, MS 38677, U.S.A.

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We have studied the viscosity dependence of the acrylamide quenching of the fluorescence on the internal tryptophan residues in cod parvalbumin and ribonuclease T_1, as well as the model systems, *N*-acetyl-L-tryptophanamide and glucagon. For the latter systems, the apparent rate constant, k_q(app), for acrylamide quenching shows a typical diffusion-limited behavior. For parvalbumin and ribonuclease T_1, however, the viscosity dependence of k_q(app) is quite different. There is little change in the k_q(app) values on increasing the bulk viscosity from 1 to 10 cP (by addition of glycerol), but a further increase from 10 to 100 cP results in a significant reduction in the k_q(app). Both an unfolding mechanism and a quencher penetration mechanism are considered to explain the results. Only the penetration mechanism is found to be consistent, and our data are interpreted as indicating that the rate-limiting step for quenching goes from being that of diffusion through the protein matrix, at low viscosity, to diffusion through the bulk solvent, at high viscosity. By also considering the Kramers' relationship in fitting our data, we are able to obtain insight regarding the coupling between internal fluctuations in the structure of the protein and motion of the bulk solvent. For parvalbumin and ribonuclease T_1, the internal dynamics are found to be very weakly coupled to the bulk.

1. Introduction

Solute quenching of the fluorescence of tryptophanyl residues in proteins has proved to be a useful kinetic probe for obtaining information about the exposure of such residues in proteins [1]. In cases where the tryptophanyl residues are located within the globular structure of a protein, contact between a solute quencher and such a residue may be limited. Some type of conformational fluctuation must be required to facilitate quencher-tryptophan contact in such cases. Examples of this are the quenching by acrylamide of the fluorescence of the single tryptophan residues in ribonuclease T_1 and cod parvalbumin [2–4].

In previous studies designed to characterize the mechanism of the acrylamide quenching of tryptophanyl residues in proteins, we found the apparent quenching rate constant, k_q(app), to be relatively independent of the viscosity of the bulk solvent for certain proteins with internal tryptophans [3]. For proteins with surface tryptophanyl residues, a greater dependence of k_q(app) on viscosity was observed, as expected for a diffusional reaction. The lack of viscosity dependence for proteins with internal tryptophanyl residues was interpreted as indicating that the rate-limiting step for quenching, in these cases, must involve fluctuations in the protein (i.e., to facilitate penetration of the quencher into the globular structure), instead of diffusion through the solvent.

These previous studies involved experiments at only one elevated viscosity, and k_q(app) values were determined from fluorescence intensity data. The present study extends investigation to a wider

* Present address: Merrell Dow Research Institute, Cincinnati, OH 45215, U.S.A.

range of viscosities, in order to reveal more completely the influence of this bulk property on the kinetics of the quenching process. As we will argue, the way in which k_q(app) depends on viscosity allows us to distinguish between different possible kinetic mechanisms for the quenching process. Also, the present study involves fluorescence lifetime quenching experiments. Quenching studies performed in this way avoid the complication of static quenching, by focusing on the dynamic quenching process [1,5].

2. Experimental

2.1. Materials

Cod parvalbumin was prepared as described earlier [4]. Ribonuclease T_1 was a gift from Dr. Frederick Walz, Jr, Kent State University. Glucagon and *N*-acetyl-L-tryptophanamide (NATA) were obtained from Sigma Chemical Co., and were used with further purification. Acrylamide was recrystallized from ethyl acetate. Spectral grade glycerol was obtained from Aldrich Chemical Co.

2.2. Methods

Fluorescence lifetime measurements were made with an SLM 4800 phase-modulation fluorometer. *p*-Terphenyl was used as a lifetime reference compound. An excitation wavelength of 295 nm (0.5 nm slit) was used for most experiments (except as noted below), and emission was observed at the wavelength of maximum fluorescence signal (8 nm slits). Fluorescence lifetimes were determined by the phase-lag method, using a modulation frequency of 18 MHz. All measurements were made at 20°C.

The Job method was used in all quenching experiments. Two solutions were placed in fluorescence cells; one cell contained the fluorophore in the appropriate buffered solution, and the other cell contained the fluorophore and the quencher acrylamide (2 M for the proteins, 0.5 M for NATA). The fluorescence lifetime of the fluorophore was measured as aliquots of the quencher-containing solution were transferred to the first cell. To achieve more viscous solutions, glycerol was added to both solutions. In the solutions containing both glycerol and acrylamide, the percent glycerol is the volume percent for the entire solution (i.e., acrylamide was substituted for water).

Lifetime quenching data were analyzed via the Stern-Volmer equation [1,5].

$$\frac{\tau_0}{\tau} = 1 + k_q\tau_0[Q] \qquad (1)$$

where τ_0 and τ are the respective fluorescence lifetimes in the absence and presence of quencher, Q, and k_q is the apparent quenching rate constant.

Absolute viscosities (η) were extrapolated from a table, of η vs. temperature and wt.% glycerol for aqueous glycerol solutions, published in Volume 43 of the Handbook of Chemistry and Physics (Chemical Rubber Co.). An Ostwald viscometer was used to determine whether the presence of acrylamide alters the relative viscosity of a particular glycerol-containing solution. We found acrylamide to reduce slightly the viscosity of very high percent glycerol solutions. The viscosity values plotted in fig. 2 are the average viscosity of the acrylamide-containing and acrylamide-free glycerol solutions.

It was found that solutions containing greater than 65% glycerol and 2 M acrylamide tended to form gels (presumably due to polymer formation) after about 1 day. We therefore used only freshly made solutions in our high glycerol content studies.

The absorbance of acrylamide in the range of 295 nm was found to increase slightly on going from water to a glycerol/water mixture. For example, the ε_{295} for acrylamide is 0.23 cm^{-1} M^{-1} in water, but 0.41 cm^{-1}/M^{-1} in approx. 75% glycerol. Because of the large absorbance of acrylamide in such high glycerol content solutions, we found it necessary to excite at slightly longer wavelengths (296–298 nm) in our fluorescence lifetime measurements with the 75–85% glycerol solutions.

3. Results and discussion

3.1. Model systems

Before going to our work with proteins, we will describe our studies of the viscosity dependence of the acrylamide quenching of the fluorescence of the model compound, *N*-acetyl-L-tryptophanamide (NATA). In previous viscosity dependence studies with such a small fluorophore, we found the acrylamide quenching rate constant, k_q, to follow the Stokes-Einstein (S-E) relationship ($k_q = 8RT/(3000\eta)$; a plot of k_q vs. T/η being linear) over a wide viscosity range, with a slight deviation possibly occurring at very low T/η. This apparent S-E behavior was obtained by changing T, by varying η in glycerol/water mixtures, or by use of various pure organic solvents [6]. We have now reinvestigated this viscosity dependence by using fluorescence lifetime Stern-Volmer plots and we have extended such studies to more viscous solutions than previously studied. Typical Stern-Volmer plots are shown in fig. 1.

Fig. 2 shows a plot of log k_q vs. log η for the acrylamide quenching of NATA. The dashed line with a slope of -1 illustrates the viscosity dependence that is predicted by the S-E equation. Clearly there is a deviation from this S-E behavior at high η (> 10 cP), although the behavior below 10 cP follows the S-E line reasonably well.

It is certainly important to understand the basis for this deviation from S-E behavior in this model system before we consider studies with proteins. It turns out that the viscosity dependence we observe for this model system is in agreement with that found for a number of similar diffusional reactions. Alwattar et al. [7] have reviewed the literature for diffusion-limited reactions and have found that in general the diffusion of small molecules in liquids does not show perfect S-E behavior. Among the reactions they considered were fluorescence quenching, excimer formation, and direct measurements of diffusion coefficients. They found that most data were well described by the following empirical relationship

$$k = AT\eta^{-1} + BT\eta^{(x-1)} \qquad (2)$$

where A, B and x are constants and k is some diffusion-limited rate constant. (Note that if B is much smaller than A and/or $x = 1.0$, this equation predicts S-E behavior.) For a number of cases examined, the values of x were found to lie in the

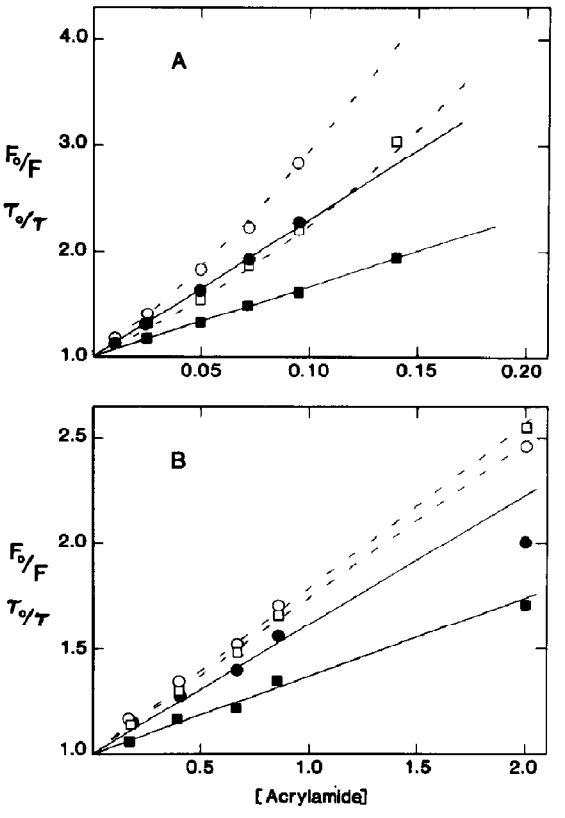

Fig. 1. Representative Stern-Volmer quenching plots. (A) Acrylamide quenching of NATA fluorescence at 20°C in water (circles) and in 50% (w/w) glycerol (squares). The solid symbols are from phase lifetime measurements (in the absence of quencher, $\tau_0 = 2.82$ and 3.57 ns for NATA in water and 50% glycerol, respectively). The solid line is a fit of eq. 1. The open symbols are from steady-state fluorescence intensity measurements and the dashed line is a fit to the modified Stern-Volmer equation (eq. 3 of ref. 6) with $K_{SV} = 13.0$ M^{-1} and $V = 2.5$ M^{-1} for water, and $K_{SV} = 6.7$ M^{-1} and $V = 3.0$ M^{-1} for 50% glycerol. (B) Acrylamide quenching of ribonuclease T_1 fluorescence in aqueous buffer (circles) and in a 50% glycerol/aqueous buffer solution (squares). Solid symbols are from lifetime measurements (in the absence of quencher, $\tau_0 = 3.0$ and 3.3 ns for ribonuclease T_1 in water and 50% glycerol, respectively). The open symbols are from fluorescence intensity measurements. The dashed line has no theoretical significance.

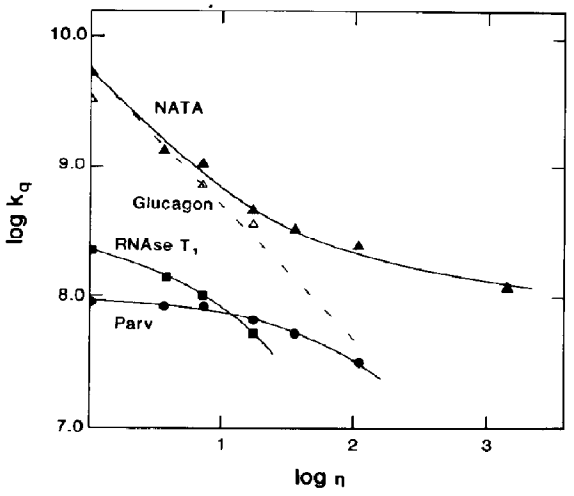

Fig. 2. Dependence of the apparent rate constant for acrylamide quenching on the bulk viscosity (in cP) for NATA (▲), glucagon (△), ribonuclease T_1 (■), and parvalbumin (●). The dashed line represents the Stokes-Einstein viscosity dependence. The solid line through the NATA data is a fit of eq. 2 with $A = 1.65 \times 10^7$ cP M^{-1} s^{-1} K^{-1}, $B = 0.96 \times 10^6$ cP M^{-1} s^{-1} K^{-1} and $x = 0.9$. The solid lines through the data for ribonuclease T_1 and parvalbumin are fits of eqs. 4 and 5 with the parameters given in table 1.

range of 0.75 ± 0.25. According to a hole diffusion theory elaborated by Gierer and Wirtz [8], the value of x can be related to the difference between the activation energy for viscous flow of the solvent and the activation energy for the migration of a solute molecule into solvent holes [7].

Our point here is not to validate eq. 2, but to indicate that the behavior we find for the acrylamide quenching of NATA fluorescence shows a viscosity dependence typical of a diffusion-limited reaction. The solid line in fig. 2 is a fit of eq. 2 with $A = 1.65 \times 10^7$ cP $(M\ s\ K)^{-1}$, $B = 0.96 \times 10^6$ cP $(M\ s\ K)^{-1}$, and $x = 0.9$. These values are similar to those found for other diffusion-limited reactions [7].

3.2. Proteins

Shown in fig. 2 is the viscosity dependence of the acrylamide quenching of the fluorescence of the single tryptophan residues of glucagon, ribonuclease T_1, and cod parvalbumin. For glucagon the viscosity dependence of k_q is similar to that for NATA. This is expected since glucagon is a polypeptide with little three-dimensional structure and its tryptophan residue is solvent exposed. (We noted no change in the peak position of the emission spectrum of glucagon on adding glycerol.)

For ribonuclease T_1 and cod parvalbumin, the viscosity dependence is quite different. In the 1–10 cP viscosity range, there is a relatively small dependence of k_q on viscosity; above 10 cP, there is a much larger dependence of k_q on viscosity. This confirms our previous reports on the small viscosity dependence for the acrylamide quenching of these proteins between 0 and 50% glycerol [2,3]. We found that the very blue emission spectra of these two proteins are unchanged in the highest glycerol-containing mixtures used.

The distinctive pattern observed for the acrylamide quenching of the internal tryptophans in these two proteins leads to two conclusions. One is that the quenching by acrylamide does not involve an appreciable amount of resonance energy transfer. The presence of glycerol increases the absorptivity of acrylamide above 295 nm. This results in an increase in the overlap integral for putative tryptophan-acrylamide energy transfer: an increase in the apparent quenching efficiency, due to this mechanism, would be expected. Our result shows just the opposite. At very high viscosity there is a decrease in the rate constant for acrylamide quenching. This drop in k_q with viscosity can only be explained in terms of a decrease in the frequency of collisions between the quencher and the internal tryptophans of these two proteins.

3.3. Kinetic models

Our results also allow us to distinguish between two possible kinetic quenching mechanisms, that involving unfolding of the protein and that involving dynamic penetration of quencher into the protein. The viscosity dependence we observe is only consistent with the latter.

These two kinetic models can be essentially represented as follows [9,10].

$$\text{P-trp*(folded)} \underset{k_f}{\overset{k_{un}}{\rightleftharpoons}} \text{P-trp*(unfolded)} \xrightarrow{k_d[Q]} \text{P-trp*(unfolded)} \cdot Q \xrightarrow{k_i} \text{quenching}$$

unfolding mechanism

$$Q + \text{P-trp*} \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (Q \ldots \text{P-trp*}) \xrightarrow{\chi} (\text{P-trp*} \cdot Q) \xrightarrow{k_i} \text{quenching}$$

penetration mechanism

where P-trp* is a protein (or other assembly) containing an excited tryptophan residue (or other fluorophore).

In the unfolding model this protein is considered to exist in two conformations, one having the residue buried from quencher, and the other having the residue solvent exposed, as a result of an unfolding of the protein's structure (this may be a local unfolding). The unfolded and folded structures interconvert with rate constants k_f and k_{un} and the equilibrium constant for this transition is $K_{unfold} = k_{un}/k_f$. Once the tryptophan residue is exposed in the unfolded structure it is quenched by diffusion of the quencher through the bulk solvent with rate constant k_d to form an encounter complex, followed by rapid deactivation with rate constant k_i. Since acrylamide has been shown to be an efficient quencher of indole fluorescence under all conditions, k_i can be considered to be very large compared to the other rate constants [11]. According to this model, the apparent rate constant for acrylamide quenching is

$$k_q(\text{app}) \approx \frac{k_{un}k_d}{k_f + k_d[Q]} \approx \frac{k_{un}}{k_f}k_d \qquad (3)$$

where the approximation on the right holds at low [Q]. (If this condition were not to hold, a Stern-Volmer plot would be downward curving, even for a single tryptophan-containing protein.) In this kinetic model, the viscosity dependence of k_q(app) should reflect that of k_d. The viscosity dependence of k_d should be given by the S-E equation, or eq. 2, as is found in our model studies. Both k_{un} and k_f should be viscosity dependent, but their ratio is expected to be relatively independent of viscosity. The addition of glycerol to aqueous protein solutions has the effect of stabilizing the native structure of some proteins [12–14], so, if anything, the ratio k_{un}/k_f may decrease slightly upon adding glycerol. Thus, the unfolding model predicts that k_q(app) should decrease with increasing viscosity, in a similar manner to that found in the model systems of NATA or the peptide glucagon. The viscosity dependence of k_q(app) for ribonuclease T_1 and cod parvalbumin is clearly different than that predicted by this model, unless one argues that the ratio k_{un}/k_f increases with viscosity.

In the penetration model, a two-step diffusional process is considered. The quencher first diffuses through the solvent, with rate constant k_d, to approach the surface of the protein (represented by (Q ... P-trp*) in the above penetration scheme). If the tryptophan residue is an internal residue, then the quencher must migrate into the globular structure of the protein to strike the tryptophan. This internal diffusion step, given by rate constant χ, will depend on the frequency of rapid fluctuations in the protein's structure, which facilitate the inward penetration of a molecule the size of the quencher. The external diffusion step (k_d) should depend on the bulk viscosity (e.g., S-E equation or eq. 2), but the internal migration step need not show a significant viscosity dependence.

The apparent rate constant for quenching, according to this penetration model, is given as

$$k_q(\text{app}) \approx \frac{\chi k_d}{\chi + k_{-d}} \approx \frac{\chi k_d}{\chi + k_d} \qquad (4)$$

(where we assume that $k_{-d} \approx k_d$ in the expression to the right; k_d and k_{-d} are both defined as diffusional steps through the solvent, in this model, and should have similar magnitude and viscosity and temperature dependences). The viscosity dependence of k_q(app) will reflect the viscosity dependence of the rate-limiting step. If $k_d < \chi$, the viscosity dependence of k_q(app) will be that of k_d. If $k_d > \chi$, the viscosity dependence of k_q(app) will be that of χ. As mentioned above, the rate constant χ may have little or no viscosity depen-

dence, and thus in the latter case ($k_d > \chi$) the experimentally observed k_q(app) will show little viscosity dependence. Parvalbumin and ribonuclease T_1 exhibit this pattern at low viscosity. Furthermore, if the rate-limiting step is changed from χ to k_d, as the bulk viscosity is increased, k_q(app) will become more viscosity dependent (i.e., it will then show the viscosity dependence of k_d).

This penetration model thus explains the viscosity dependence we observe for the acrylamide quenching of the internal tryptophans in parvalbumin and ribonuclease T_1.

A fit of eq. 4 to the data in fig. 2 can be achieved by allowing each rate constant, k_d and χ, to have particular viscosity dependences, according to the following simplified version of the Kramers' equation.

$$k_{(k_d \text{ or } \chi)} = \frac{A}{\eta^x} \exp\left(-\Delta H^{\ddagger}/RT\right) = \frac{k^0}{\eta^x} \qquad (5)$$

where A and $\Delta H^{\ddagger}$ are the usual Arrhenius parameters and k^0 is the rate constant at the experimental temperature and at a viscosity of 1 cP. In this equation the bulk viscosity is raised to some power x (between 0 and 1.0).

The above version of the Kramers' equation has recently been found useful for interpreting the effect of bulk viscosity on rate processes involving proteins [15–17]. A value of $x = 1.0$ in the above equation means that the rate constant is inversely dependent on the bulk viscosity; a value of $x = 0$ means that the rate constant is completely independent of viscosity.

Fig. 2 shows fits, using a combination of the Kramers' equation (eq. 5) and the penetration model (eq. 4), to the quenching data for parvalbumin and ribonuclease T_1. Table 1 summarizes the fitting parameters. Good fits are obtained with small values of x for χ and large values of x for k_d. This is consistent with the above expressed notion that the internal diffusion process (χ) need not be viscosity dependent. This step involves movement of the quencher into and through the protein matrix and such movement will be viscosity dependent only to the extent that the fluctuations in the structure of the protein are coupled to the viscosity of the surrounding medium. The fact that x is found to be slightly above zero suggests that some dynamic coupling does exist between the protein and the bulk, but that this coupling is weak.

Table 1

Parameters used to fit the penetration model and Kramers' equation to data for the viscosity dependence of the acrylamide quenching of parvalbumin and ribonuclease T_1[a]

Protein	External diffusion step		Internal diffusion step	
	k_d ($\times 10^9$ M^{-1} s^{-1})	x	χ ($\times 10^{-9}$) (s^{-1})	x
Parvalbumin	2	0.8	0.1	0
Ribonuclease T_1	1	0.9	0.3	0.1

[a] Fits to the data in fig. 2 using eqs. 4 and 5. The k_d and χ values given here refer to their values at $\eta = 1$ cP and 20°C.

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