Fluorescence Quenching of Buried Trp Residues by Acrylamide Does Not Require Penetration of the Protein Fold

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The accessibility of acrylamide to buried Trp residues in proteins, as attested by dynamic quenching of their fluorescence emission, is often interpreted in terms of migration of the quencher (Q) through the globular fold. The quencher penetration mechanism, however, has long been debated because, on one hand, solutes the size of acrylamide are not expected to diffuse within the protein matrix on the nanosecond time scale of fluorescence and, on the other hand, alternative reactions pathways where Q remains in the solvent cannot be ruled out. To test the Q penetration hypothesis, we compared the quenching rates of acrylamide analogs of increasing molecular size (acrylonitrile, acrylamide, and bis-acrylamide) on the buried Trp residues of RNaseT1 and parvalbumin. The results show that the largest molecule, bis-acrylamide, is also the most efficient quencher and that in general the quenching rate is not correlated to quencher size, as expected for a penetration mechanism. Whereas these results rule out significant internal Q migration in the times of fluorescence, it is also demonstrated that up to a depth of burial of 3 Å, through-space interactions with acrylamide in the solvent satisfactorily account for the small rate constants reported for these proteins. More generally, this analysis emphasizes that reduced dynamic quenching of protein fluorescence by acrylamide rather than reporting on the structural rigidity of the globular fold reflects the distance of closest approach between the internal chromophore and Q in the aqueous phase.

Introduction

Starting with the seminal work of Lehrer,[1] quenching of Trp fluorescence and phosphorescence by added small solutes has been widely employed in structural studies because it can provide information on the location of Trp residues with respect to the aqueous phase and on the permeability of proteins to solutes of various molecular size.[2−5] The accessibility of quenchers to internal Trp residues has been instrumental for probing the flexibility of these macromolecules,[2,4−11] although for large solutes like acrylamide, interpretation of quenching rates in terms of the frequency and amplitude of structural fluctuations may not be warranted.[9,12,13]

Quenching of Trp fluorescence by acrylamide is one of the most thoroughly investigated systems and is still widely applied in biophysical studies of protein structure. Fluorescence quenching experiments measure either the fluorescence intensity (F) or the fluorescence lifetime (τ), or both, as a function of the quencher concentration, [Q], and evaluate the bimolecular quenching rate constant, k_q, from the gradient of the Stern−Volmer plot

$$F_0/F = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad \text{or} \quad \tau_0/\tau = 1 + k_q\tau_0[Q]$$

where F_0 and τ_0 are the unperturbed intensity and lifetime, respectively. With Trp residues buried inside proteins, k_q is considerably smaller than the diffusion-limited rate constant ($k_d = 4\pi r_0 D$, r_0 is the sum of molecular radii and D is the sum of the diffusion coefficients) and in some instances falls below the detection limit ($\sim 5 \times 10^7$ M^{-1} s^{-1}). Until now, a ratio $k_q/k_d \ll 1$ has often been interpreted as the hindered diffusion of Q through the globular fold and therefore as a measure of its structural flexibility.[2,14] For k_q to reflect the permeability of a protein to Q, quenching of buried Trp residues must be limited by the slow migration of Q through the protein matrix. However, whereas it is generally accepted that small neutral diatomic (O_2, CO, and NO) and to a lesser extent triatomic molecules (H_2S, CS_2) can migrate through the protein matrix on the nanosecond time scale of fluorescence, the debate is open for larger solutes such as acrylamide.[9,12,13,15] For molecules of this size and larger ones, it is argued that Q penetration is most unlikely as the globular fold tends to be compact with rather few and small cavities, which render it largely impermeable. This point of view is also supported by a molecular dynamics simulation[16] that predicted a sharp dependence of protein penetration rate on molecular size. Furthermore, there is the possibility of alternative quenching mechanisms, involving either long-range through-space interactions between Q and chromophore[6,13] or transient partial unfolding of the polypeptide bringing the chromophore to the surface,[6,12,13] both of which would permit efficient quenching by Q in the solvent. Indeed, the demonstration that the interaction between acrylamide and the fluorescent state of indole does not require physical contact between reactants[17] entails that superficially buried Trp's could directly interact with acrylamide in the solvent. Additional ambiguities with the interpretation of k_q in terms of hindered diffusion may arise from potential artifacts linked to the propensity of acrylamide to bind to proteins.[18] Binding may result in partitioning of acrylamide into proteins and in worst cases may even cause perturbations of the native structure.

The penetration mechanism has been advocated mainly to account for specific features of acrylamide quenching of the single Trp proteins RNaseT1 and parvalbumin.[2,14] The indole ring in these proteins is fully buried within the globular fold,

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separated from the solvent by a 2 to 3 Å thick protein spacer, and k_q is ~30 fold smaller (~10^8 M^{-1} s^{-1}) than anticipated for solvent exposed Trp. The experimental evidence deemed crucial in supporting Q penetration is that: (1) external quenching would be unable to explain the relative invariance of k_q to the change in solvent viscosity, (2) the activation energy for k_q is larger than that for acrylamide diffusion in the aqueous phase, and (3) the greater reduction of k_q for succinamide, which is a larger, although less efficient, quencher than acrylamide. Undoubtedly, a decisive test for discriminating quencher penetration from alternative quenching pathways is the dependence of the rate on quencher size. This issue was first pursued by Calhoun et al.,[12] who determined the rate of fluorescence quenching exhibited by moieties differing in molecular size, polarity, and charge on a set of proteins that cover a whole range of Trp accessibility. The results on RNaseT1 and parvalbumin, as well as on other proteins that displayed measurable quenching, showed that the disparate quenchers studied exhibited rather similar rate constants. Because such an outcome is not consistent with quencher penetration, it was concluded that the reaction pathway presumably involves Q encounters with Trp's that become occasionally exposed to the solvent. The above comparison among quenchers, however, does not provide an unambiguous test of the dependence of the quenching rate on quencher size because different quenching moieties may exhibit dissimilar interaction ranges. To avoid the above ambiguity, we investigated the rate of fluorescence quenching of RNaseT1 and parvalbumin by neutral acrylonitrile, acrylamide, and bis-acrylamide analogs in which the quenching moiety is either similar to or identical to acrylamide, whereas the molecular weight ranges from 53 to 154 Da. The results demonstrate that k_q is not correlated to quencher size and thus confirm that on the nanosecond time scale of fluorescence a molecule the size of acrylamide is precluded from penetrating even superficial regions of these macromolecules.

Experimental Methods

All chemicals were of the highest purity grade available from commercial sources and, unless specified on the contrary, were used without further purification. Tris(hydroxymethyl)aminomethane (Tris) and $CaCl_2$ Suprapur were from Merck (Darmstadt, Germany). The protein parvalbumin from whiting fish was purchased from Calbiochem Corporation (San Diego, CA), and ribonuclease T1 (RT1) was a kind gift from Dr. Nick Pace (Texas A&M University). Acrylamide (99.9% electrophoretic purity) was from Bio-Rad Laboratories (Richmond, CA). *N*,*N*′-methylenbisacrylamide (99%), acrylonitrile (99%), and *N*-acetyltryptophanamide (≥99%) were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified by reverse osmosis (Milli-RX 20, Millipore, Billerica, MA) and subsequently passed through a Milli-Q Plus system (Millipore, Bedford, MA). RNaseT1 was dialyzed into 50 mM Tris (pH 7) and parvalbumin was dialyzed into 50 mM Tris, 10 mM $CaCl_2$ (pH 8). Quencher stocks were prepared daily by dissolving commercial supplies in the experimental buffer.

Fluorescence Quenching Experiments. Steady-state fluorescence spectra and intensities were measured on a commercial fluorometer, Perkin-Elmer (Waltham, MA) LS50B, using an excitation wavelength of 295 nm (2 nm slits) in all studies. To avoid the Raman peak, the emission intensity was monitored at 320 nm with the proteins and at 350 nm with NATA using a 10 nm slit. The concentration of proteins and of NATA was adjusted to have an absorbance of ~0.05 at the excitation wavelength.

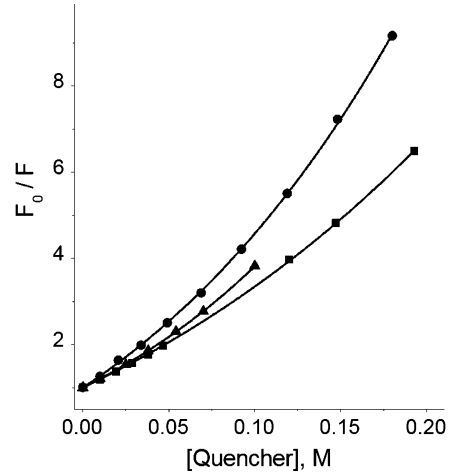

Figure 1. Stern−Volmer plots for the quenching of NATA fluorescence by acrylonitrile (●), acrylamide (■), and bis-acrylamide (▲) in 50 mM Tris, pH 7, at 20 °C. λ_{ex} = 295 nm and λ_{em} = 350 nm.

Acrylamide and acrylonitrile quenching studies were performed by the addition of aliquots of a stock quencher solution to a 5 × 5 mm^2 cell containing the protein/NATA sample. In the case of bis-acrylamide, which has limited solubility in water, the quencher concentration was varied by mixing two solutions identical in protein or NATA concentration, one containing concentrated bis-acrylamide and the other being quencher-free. The fluorescence background, measured in parallel experiments where the chromophore was omitted, was generally small and was subtracted from sample data before inner filter corrections were applied. In the case of acrylamide and bis-acrylamide, inner filter correction was applied as described before[19] using ε_{295} = 0.25 and 1.22 M^{-1} cm^{-1} for acrylamide and bis-acrylamide, respectively. Practically no correction was necessary for acrylonitrile.

Quenching rate constants were computed from a modified Stern−Volmer equation[2]

$$F_0/F = (1 + K_{SV}[Q])e^{V[Q]} \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence of quencher and in the presence of quencher at a concentration [Q], respectively. V is the static quenching constant and K_{SV} is the dynamic quenching constant. The second-order quenching rate constant, k_q, is calculated as $k_q = K_{SV}/\tau_0$, where τ_0 is the unperturbed fluorescence lifetime reported for these Trp residues in the literature.

All experiments were carried out at 20 °C.

Results

Quenching of NATA Fluorescence by Acrylonitrile and Bis-acrylamide. NATA is the Trp derivative usually taken as the reference state for Trp residues in peptides and proteins that are fully exposed to the solvent. Figure 1 reports the Stern−Volmer plots for the quenching of NATA fluorescence by acrylonitrile, acrylamide, and bis-acrylamide in 50 mM phosphate buffer at 20 °C. In the case of bis-acrylamide, the concentration range is limited by the solubility of the compound. Each plot deviates from a straight line exhibiting a distinct upward curvature. The data was fitted to eq 1, where the constant V (the active volume) takes into account contributions from static quenching and transient effects[2] arising from quenchers in the proximity of the chromophore at the time of excitation. The values of K_{SV}, V, and the second-order rate constant $k_q = K_{SV}/$

TABLE 1: Parameters Relative to the Fluorescence Quenching of NATA and of the Proteins RNaseT1 and Parvalbumin by Acrylonitrile, Acrylamide, and Bis-acrylamide at 20 °C

sample	quencher	K_{SV} M^{-1}	V M^{-1}	τ_0 ns	$k_q \times 10^{-9}$ M^{-1} s^{-1}
NATA	acrylonitrile	23.2	3.2	3.0	7.7
	acrylamide	16.8	2.2	3.0	5.6
	bis-acrylamide	16.0	3.9	3.0	5.3
RNaseT1	acrylonitrile	0.93	0	3.6[27]	0.26
	acrylamide	1.1	0	3.6	0.31
	bis-acrylamide	2.1	0	3.6	0.58
parvalbumin	acrylonitrile	2.1	0	4.6[28]	0.46
	acrylamide	1.0	0	4.6	0.22
	bis-acrylamide	3.2	0	4.6	0.70

τ_0 are collected in Table 1. The results obtained with acrylamide are in good agreement with published data. Acrylonitrile and bis-acrylamide are slightly more efficient that acrylamide, although the value of k_q is similar among the three quenchers and approaches closely the diffusion controlled rate, k_d ($k_d = 7.5 \times 10^9$ M^{-1} s^{-1}).[2] The main difference among the quenchers is the magnitude of the static component, V, increasing from 2.2 $M^{-1}$for acrylamide to 3.2 M^{-1} for acrylonitrile and to 3.9 M^{-1} for bis-acrylamide. To a first approximation, the value of V of bis-acrylamide is expected to double. The reason is that upon assuming an unaltered quencher distribution about NATA, the concentration of quenching groups in proximity to the chromophore giving rise to static quenching is twice as large in the case of bis-acrylamide. For acrylonitrile, the increase in V could derive from a longer-range quenching interaction for this analog or from a preferential distribution to the vicinity of the chromophore. The conclusion from the above findings is that in aqueous solutions, both acrylamide analogs quench NATA fluorescence very efficiently and that k_q is limited by the rate of quencher diffusion to the chromophore.

Fluorescence Quenching of Parvalbumin and RNaseT1. Quenching of whiting fish Ca^{2+}-parvalbumin and of RNaseT1 fluorescence by acrylamide and its analogs was carried out in buffer at 20 °C. Up to the highest concentration examined, the addition of these quenchers did not alter the absorption or the fluorescence spectrum of the proteins that might indicate a perturbation of the native fold. The Stern−Volmer plots obtained from the decrease in fluorescence intensity, shown in Figure 2, are in every case linear on the quencher concentration. The values of K_{SV} derived from the gradient of these plots are collected in Table 1 together with the average fluorescence lifetime (τ_0) reported in the literature for these proteins. Also listed in the Table is the apparent quenching rate constant calculated from the relationship $k_q = K_{SV}/\tau_0$. The term apparent reflects the fact that intensity measurements cannot distinguish between dynamic and static quenching, especially when the reaction is inefficient. Indeed, with acrylamide quenching, the gradient of the lifetime (τ_0/τ) plot was smaller than that of the corresponding intensity plot in both proteins, revealing a non-negligible static contribution, between 30 and 50%, to the overall quenching rate.[14,20] Therefore, by assuming K_{SV} to be 100% dynamic, the k_q values of Table 1 are overestimates, merely representing the upper bounds of dynamic quenching. It will become apparent that the above distinction is not important for the purpose of the present investigation, namely, the demonstration that quencher penetration of the protein does not occur on the nanosecond time scale of fluorescence.

For acrylamide, K_{SV} is 1.1 for RNaseT1 and 1.0 M^{-1} for parvalbumin, respectively, values that are in substantial agreement with previous reports,[14,20] even if for parvalbumin, are is available only for the protein derived from cod fish rather than from whiting fish. (Whiting fish parvalbumin is structurally very similar to that from cod and carp, with a sequence homology of ~80%. The crystal structures, available only for whiting fish and carp, match very well in terms of secondary structure elements and exhibit an alignment of 104 residues (Cα) over 108 and a rmsd of 1.02 Å. The region about W102 is highly conserved, and the separation of the indole ring from the aqueous interface is practically identical in the two structures.) With both proteins, K_{SV} is reduced about 17-fold relative to NATA, and k_q is reduced by a factor at least as large. From a quencher penetration perspective, this factor would represent the extent to which acrylamide diffusion through the protein matrix is slowed down relative to diffusion in water. However, bis-acrylamide that is more than twice the size of the monomer is actually a more efficient quencher than acrylamide; K_{SV} increases two times with RNaseT1 and three times with parvalbumin. The smallest quencher, acrylonitrile, behaves differently with the two proteins. Relative to acrylamide, its K_{SV} increases two-fold with parvalbumin, but it actually decreases by ~20% with RNaseT1. The key conclusion from the present results is that in both proteins, the quenching efficiency of these acrylamide analogs ranging in M_w from 53 to154, even after normalization for the effective concentration of quenching moieties, hardly correlates with molecular size, as would be expected for a quencher penetration mechanism. Indeed, for RNaseT1, the quenching rate increases with quencher size a trend just opposite of that anticipated.

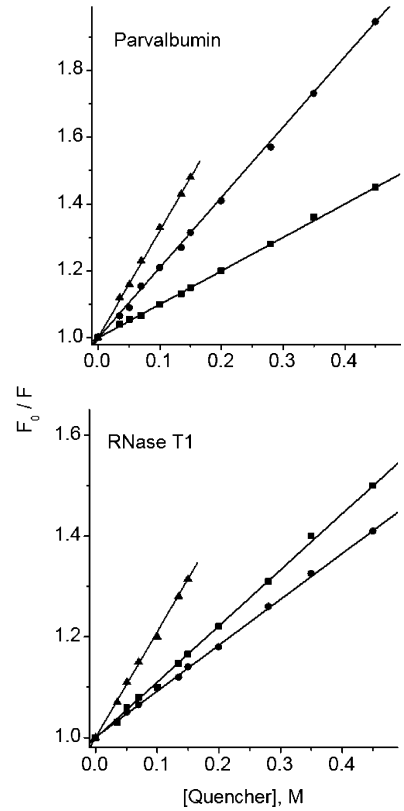

Figure 2. Top panel: Stern−Volmer plots for the quenching of Ca^{2+}-parvalbumin fluorescence by acrylonitrile (●), acrylamide (■), and bis-acrylamide (▲) in 50 mM Tris +2 Mm CaCl, pH 8. Bottom panel: Stern−Volmer plots for the quenching of RNaseT1 fluorescence by acrylonitrile (●), acrylamide (■), and bis-acrylamide (▲) in 50 mM Tris, pH 7. $\lambda_{ex} = 295$ nm and $\lambda e_m = 320$ nm. The temperature is 20 °C.

Discussion

Evidently, the lack of a correlation between quencher size and quenching rate is not compatible with a penetration mechanism. Possible alternative interpretations of protein fluorescence quenching involve either direct contact between chromophore and quencher following transient exposure of Trp to the protein surface or long-range, through-space interactions between the buried chromophore and Q in the aqueous phase. A number of arguments rule out the possibility that major structural rearrangements do occur on the fluorescence time scale to expose transiently the indole ring to the solvent,[9,21] as will be mentioned below. Long-range external quenching can be either dynamic or static, or both, depending on whether it involves, respectively, collision with or binding of Q to the protein surface, in proximity of the chromophore. We contend that a combination of these routes can satisfactorily account for the lack of a correlation between quenching rate and Q size, reported above, as well as for the peculiar viscosity and temperature dependence of acrylamide quenching in these proteins reported in the literature.

Static Quenching from Q Binding. According to the protein's crystallographic structure, the indole rings of W59 in RNaseT1[22] and of W102 in parvalbumin (Declercq, J. P.; Baneres, J. L.; Rambaud, J.; Parello, J.; PDB code: 1a75) are both tightly packed within a hydrophobic pocket separated from the solvent by a protein spacer, which, in the region nearest to the aqueous interface, is 2 to 3 Å thick. The distance dependence of the quenching interaction between acrylamide and the fluorescent state of Trp was found to be[17] $k(r) = (6 \times 10^{13})$ $\exp(-r/0.32)$ s^{-1}, where r (Å) is the net separation between reactants (the distance beyond van der Waal's contact). Therefore, acrylamide bound to the protein surface at a separation of 2.5 Å from the indole ring will quench its fluorescence at a rate of $k_b = 2.4 \times 10^{10}$ s^{-1}. More generally, this quenching route will be effective for acrylamide molecules that bind to within 3.5 Å of the chromophore. For a single binding site at a distance r', the static contribution to the overall quenching rate is given by

$$f_b k_b(r') = [1/(K_d/[Q] + 1)]k_b(r') \quad (2)$$

where $f_b = 1/(K_d/[Q] + 1)$ is the Q-bound protein fraction and K_d is the corresponding dissociation constant. Because molar, submolar acrylamide affinities are not uncommon with proteins, binding could make a significant contribution to the overall quenching rate, especially when large [Q] values are employed. Binding could thus account for the smaller gradient of lifetime Stern−Volmer plots relative to the corresponding intensity plots and for the observation of very short-lived components in the fluorescence decay in the presence of acrylamide.[15]

Dynamic Quenching. Pertinent analytical expressions for dynamic quenching of buried Trp residues by Q diffusion to the protein surface, followed by long-range interactions with the buried chromophore, have been discussed in detail before.[23] The steady-state quenching rate constant, obtained by solving Fick's diffusion equation applying radiation boundary conditions, neglecting a transient time-dependent term arising from Q molecules already in the proximity of the chromophore at the instant of excitation (here incorporated in the static component), is given by

$$k_q = k_D k_I/(k_D + k_I) \quad (3)$$

where $k_D = 4\pi aD$ (cm^2 s^{-1}) is the Smoluchowski diffusion-controlled rate constant, $k_I = 4\pi a^2 B$ (cm^2 s^{-1}) is the diffusion-independent rate constant (the rapid diffusion limit), and D is the sum of the separate diffusion coefficients of Q and protein in solution. B is an interaction strength parameter related to the distance dependence of the quenching interaction, $k(r)$, and a is the distance of the closest approach between the internal chromophore and Q in the solvent. For a quenching interaction of the form $k(r) = k_0 \exp(-r/\lambda)$, the diffusion-independent rate constant, k_I (assuming an essentially flat protein surface),[24] is given by

$$k_I(a) = 2\pi N k_0 10^{-3}[\lambda a^2 + 2\lambda^3] \exp(-a/\lambda) \quad M^{-1}s^{-1} \quad (4)$$

where N is Avogadro's number and the distances a and λ are in centimeters.

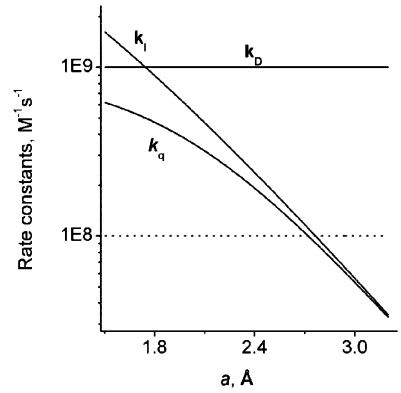

Figure 3. Theoretical estimate of the through-space acrylamide quenching rate constant (k_q) in a protein as a function of the distance of closest approach (a) between buried Trp and Q in the solvent based on eqs 3 and 4 assuming a value of $k_D = 10^9$ M^{-1} s^{-1}. The dotted line shows that the experimental value of $k_q \approx 10^8$ M^{-1} s^{-1} is consistent with a separation of 2.7 Å.

The variation of k_I with the distance of closest approach (a), using eq 4 and $k(a) = (6 \times 10^{13}) \exp(-a/0.32)$ s^{-1}, is shown in Figure 3 together with the corresponding variation of k_q predicted by eq 3 for a value of $k_D = 10^9$ M^{-1} s^{-1}. Figure 3 illustrates two important points: (i) The experimental value of k_q ($\sim 10^8$ M^{-1} s^{-1}) representing the dynamic component of acrylamide quenching of RNaseT1 and parvalbumin is attained for a separation $a \approx 2.7$ Å, which is consistent with crystallographic data. Therefore, through-space dynamic quenching with acrylamide in the solvent provides a viable mechanism to quench the fluorescence of superficially buried Trp residues. In practice, quenching by this route is measurable ($k_q \geq 5 \times 10^7$ M^{-1} s^{-1}) up to a maximum separation between indole and the solvent interface of $\sim$3 Å. It may be significant that more deeply buried Trp residues, such as W314 of LADH ($a = 4.5$ Å) and W48 of azurin ($a = 8$ Å), are completely inaccessible to acrylamide quenching.[2,25] (ii) Increasing the distance of closest approach, k_I drops rapidly, and the reaction that at short-range is under the diffusion-control regime ($k_D < k_I$) progressively shifts to the reaction-control regime ($k_D > k_I$). For $k_D = 10^9$ M^{-1} s^{-1}, the transition midpoint is at $a \approx 1.8$ Å. At the distance of 2.7 Å, when k_q coincides with the experimental value, $k_D/k_I \approx 10$ and the reaction approaches the rapid diffusion limit. In the latter regime, k_q is invariant to diffusion and will therefore be insensitive to both Q size and to changes in solvent viscosity (η_s). Only in more viscous solution (when $k_D < k_I$) will k_q gradually acquire the full linear dependence on D, as was reported in the literature for these proteins. Therefore, quenching near the rapid diffusion limit, first proposed by Calhoun et al.[8] as a possible explanation for the observed insensitivity of k_q to solvent viscosity, provides a rational explanation for the peculiar

η_s dependence of k_q of these proteins as well as for the independence of k_q on the size of the above acrylamide analogs. It also explains the observation that bis-acrylamide is about twice as efficient a quencher as acrylamide. The reason is simply because for bis-acrylamide, the quencher concentration is twice the solute concentration, and its effect is to double the value of k_I. Another feature that has been considered to provide strong support for the quencher penetration mechanism is the activation energy of k_q, which is generally larger (~8 kcal/mol) than the 3−5 kcal/mol that is expected for diffusion in water. In the rapid diffusion regime, however, the temperature dependence of k_q is not governed by the barrier to Q diffusion in water but by the temperature dependence of k_I.

The alternative possibility that very large amplitude structural fluctuations occur on the nanosecond time scale, admitting equally solutes of such disparate size to the protein interior or transiently exposing the indole ring to the solvent, is directly ruled out by the small acrylamide phosphorescence quenching rates (^Pk_q) observed with both proteins. Indeed, should acrylamide quench both fluorescence and phosphorescence by physical contact with the chromophore, then the rate constant for fluorescence and phosphorescence quenching would necessarily have to be of similar magnitude. However, in the case of RNaseT1, $^Pk_q = 5.9 \times 10^4\ M^{-1}\ s^{-1}$,[9] whereas for parvalbumin, $^Pk_q = 2.2 \times 10^4\ M^{-1}\ s^{-1}$ (Strambini, unpublished results). Even allowing for a spin statistical factor of 1/9 for the interaction with the triplet state, these rates are more than two orders of magnitude smaller than the corresponding fluorescence quenching rate. According to ^Pk_q, acrylamide access to the protein interior is slow even on the microsecond time scale. The implication is that fluorescence quenching by acrylamide penetration is bound to be negligible not only with these proteins but also with polypeptides whose fold is much more flexible than those of RNaseT1 and parvalbumin. The discrepancy between fluorescence and phosphorescence quenching rates is generally large even for other proteins and quencher moieties,[7,9] suggesting that the role of long-range interactions in fluorescence quenching is often underestimated.

Conclusions

The lack of a correlation between quenching rate and quencher size reported here with acrylamide analogs provides a decisive test against the quencher penetration mechanism. In addition, the above analysis of acrylamide quenching demonstrates that long-range interactions with acrylamide in the solvent afford a viable mechanism to quench the fluorescence of Trp residues buried within 3 Å of the molecular surface. Until now, the accessibility of acrylamide (k_q) has been variably interpreted as either the extent of solvent exposure of the indole ring or a measure of the permeability of the local fold to the quencher.[26] The above description of the quenching process emphasizes that reduced dynamic quenching of protein fluorescence by acrylamide is not related to the structural rigidity of the local fold but, rather, is a function of the distance of closest approach between the internal chromophore and Q in the aqueous phase.

References and Notes

(1) Lehrer, S. S. *Biochemistry* **1971**, *10*, 3254.
(2) Eftink, M. R. Topics in Fluorescence Spectroscopy. In *Principles*; Lakowicz, J. R., Ed.; Plenum: New York, 1991; Vol. 2, pp 53.
(3) Eftink, M. R.; Ghiron, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3290.
(4) Lakowicz, J. R.; Weber, G. *Biochemistry* **1973**, *12*, 4171.
(5) Vanderkooi, J. M. Topics in Fluorescence Spectroscopy. In *Biochemical Applications*; Lakowicz, J. R., Ed.; Plenum: New York, 1991; Vol. 3. pp 113.
(6) Calhoun, D. B.; Vanderkooi, J. M.; Englander, S. W. *Biochemistry* **1983**, *22*, 1533.
(7) Strambini, G. B. *Biophys. J.* **1987**, *52*, 23.
(8) Calhoun, D. B.; Englander, S. W.; Wright, W. W.; Vanderkooi, J. M. *Biochemistry* **1988**, *27*, 8466.
(9) Cioni, P.; Strambini, G. B. *J. Am. Chem. Soc.* **1998**, *120*, 11749.
(10) Strambini, G. B.; Gonnelli, M. *Biochemistry* **2009**, *48*, 7482.
(11) Wright, W. W.; Owen, C. S.; Vanderkooi, J. M. *Biochemistry* **1992**, *31*, 6538.
(12) Calhoun, D. B.; Vanderkooi, J. M.; Holtom, G. R.; Englander, S. W. *Proteins* **1986**, *1*, 109.
(13) Chen, L. X.; Longworth, J. W.; Fleming, G. R. *Biophys. J.* **1987**, *51*, 865.
(14) Eftink, M. R.; Hagaman, K. A. *Biophys. Chem.* **1986**, *25*, 277.
(15) Somogyi, B.; Punyiczki, M.; Hedstrom, J.; Norman, J. A.; Prendergast, F. G.; Rosenberg, A. *Biochim. Biophys. Acta* **1994**, *1209*, 61.
(16) Richards, F. M. *Carlsberg Res. Commun.* **1979**, *44*, 47.
(17) Lakowicz, J. R.; Zelent, B.; Gryczynski, I.; Kusba, J.; Johnson, M. L. *Photochem. Photobiol.* **1994**, *60*, 205.
(18) Punyczki, M.; Norman, J. A.; Rosenberg, A. *Biophys. Chem.* **1993**, *47*, 9.
(19) Parker, C. A. *Photoluminescence of Solutions*; Elsevier: New York, 1968.
(20) Eftink, M. R.; Hagaman, K. A. *Biophys. Chem.* **1985**, *22*, 173.
(21) Eftink, M. R.; Ghiron, C. A. *Biophys. J.* **1987**, *52*, 467.
(22) Gohda, K.; Oka, K.; Tomita, K.; Hakoshima, T. *J. Biol. Chem.* **1994**, *269*, 17531.
(23) Owen, C. S.; Vanderkooi, J. M. *Comments Mol. Cell. Biophys.* **1991**, *7*, 235.
(24) Vanderkooi, J. M.; Englander, S. W.; Papp, S.; Wright, W. W.; Owen, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5099.
(25) Eftink, M. R.; Selvidge, L. A. *Biochemistry* **1982**, *21*, 117.
(26) Anbazhagan, V.; Damai, R. S.; Paul, A.; Swamy, M. J. *Biochim. Biophys. Acta* **2008**, *1784*, 891.
(27) Eftink, M. *Biophys. J.* **1983**, *43*, 323.
(28) Castelli, F.; White, H. D.; Forster, L. S. *Biochemistry* **1988**, *27*, 3366.

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