Acrylamide Quenching of Protein Phosphorescence as a Monitor of Structural Fluctuations in the Globular Fold

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Abstract: This study examines acrylamide quenching of tryptophan room-temperature phosphorescence in proteins and the role that factors such as long-range interactions and environment-dependent quenching efficiency might play in the interpretation of bimolecular quenching rate constants in terms of hindered quencher migration through the globular fold. The distance dependence of the through-space quenching rate is evaluated by studying the effects of acrylamide on the phosphorescence intensity and decay kinetics of the indole analogue 2-(3-indoyl)ethyl phenyl ketone in propylene glycol/buffer glasses, at 120 K. Both steady-state and kinetic data are satisfactorily fitted by an exponential distance dependence of the rate, $k(r) = k_0 \exp[-(r - r_0)/r_e]$, with a contact rate $k_0 = 1.2 \times 10^8$ s^{-1} and an attenuation length $r_e = 0.29$ Å. For a phosphorescence lifetime of 5 s, this rate yields an average interaction distance of 10 Å. The rate is temperature dependent, with k_0, estimated from the bimolecular quenching rate constant ($^{P}k_q$) of Trp analogues in liquids, increasing by about 10-fold from 120 to 293 K. Solvent effects on the quenching efficiency are tested with Trp analogues in water, propylene glycol, and dioxane. The quenching efficiency per collisional encounter is about 0.20 for water, 0.35 for propylene glycol, and drops to 0.025 in the aprotic, least polar dioxane. Acrylamide quenching rate constants are determined for a series of proteins and for experimental conditions appositely selected to test the importance of factors such as the degree of Trp burial and structural rigidity. Relative to $^{P}k_q = 1.5 \times 10^9$ M^{-1} s^{-1} for Trp in the solvent, the magnitude of $^{P}k_q$ for protected Trp residues in proteins ranges from a maximum of 6×10^4 M^{-1} s^{-1}, for the most superficial W59 of RNase T$_1$, to 10^{-1} M^{-1} s^{-1} for the most internal W109 of alkaline phosphatase. For most proteins, theoretical estimates of $^{P}k_q$ based on the distance dependence of the rate exclude any quenching contribution from through-space interactions by acrylamide in the solvent. This finding, together with a clear correlation between $^{P}k_q$ and other indicators of molecular flexibility, implies that in the millisecond-second time scale of phosphorescence acrylamide can migrate through the macromolecule and that its rate is a measure of the frequency and amplitude of the structural fluctuations underlying diffusional jumps. The origin of the discrepancy between fluorescence and phosphorescence quenching rates in proteins is discussed, and an alternative interpretation of fluorescence quenching data is provided.

Introduction

Quenching of Trp fluorescence and phosphorescence in proteins by added small solutes is widely employed in structural studies, for it can provide information on the location of Trp residues with respect to the aqueous phase and on the permeability of proteins to ligands of various molecular sizes.[1−5] Furthermore, quencher accessibility has been useful for revealing changes in the conformation of the polypeptide but, more importantly, also for investigating on the nature of its dynamical structure, that is, the frequency and amplitude of structural fluctuations that permit diffusion of solutes through generally well-packed and extensively bonded internal regions of the globular fold.[2,6−11]

Quenching studies measure the luminescence lifetime (τ) as a function of quencher concentration, [Q], and evaluate the bimolecular quenching rate constant, k_q, from the gradient of the Stern−Volmer plot,

$$1/\tau = 1/\tau_0 + k_q[\mathrm{Q}]$$

where τ_0 is the unperturbed lifetime. With chromophores free in solution and efficient quenching reactions, k_q is practically identical to the diffusion-limited rate constant k_d, where $k_d = 4\pi r_0 D$ (r_0 is the sum of molecular radii and D is the sum of the diffusion coefficients). With Trp residues inside proteins, depending on the nature of the quencher and on the surface accessibility of the chromophore, k_q is invariably smaller than k_d and may even fall by several orders of magnitude.[5,8,10]

In order 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. Until now, a ratio

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$k_q/k_d \ll 1$ has often been interpreted as hindered diffusion of Q through the globular fold. This interpretation, however, has been questioned, especially for large solutes because, in principle, other mechanisms, based on long-range interactions or transient partial unfolding of the polypeptide bringing the chromophore to the surface, may allow efficient quenching from Q in the aqueous phase. In fact, while it is generally accepted that small neutral diatomic (O_2, CO, and NO) and, to a lesser extent, even triatomic molecules (H_2S, CS_2, ...) can migrate through the protein matrix in the nanosecond time scale of fluorescence, the debate is open for larger solutes such as acrylamide.[4,10,12] The evidence in support of acrylamide penetration is indirect and is based mainly on the insensitivity of ${}^{F}k_q$ (the fluorescence quenching rate constant) to change in solvent viscosity, activation energies that are sensibly larger than those for acrylamide diffusion in the aqueous solvent, and a greater reduction of k_q for larger quenchers, such as succinamide.[4]

Ambiguities with the interpretation of acrylamide ${}^{F}k_q$ in terms of hindered diffusion may arise both from uncertainties with regard to the quenching mechanism and from potential artifacts linked to the propensity of acrylamide to bind to proteins.[11,12] Binding may result in partitioning of acrylamide into proteins and, in worst cases, may even cause perturbations of the native structure.[6,13] With regard to the mechanism, the recent demonstration that an effective interaction between acrylamide and the fluorescent state of indole does not require physical contact between the reactants[14] points out that superficially buried Trps, namely within 4−5 Å of the aqueous interface, can be directly quenched by acrylamide in the solvent. In this respect, it may be significant that fluorescence quenching by acrylamide has not been observed in protein with more deeply buried Trp residues. Further, phosphorescence quenching studies with buried W314 of liver alcohol dehydrogenase (LADH) by quenchers the size of acrylamide and larger suggest that their migration through the protein is negligible in the nanosecond time scale. Based on the relative invariance of ${}^{P}k_q$ (the phosphorescence quenching rate constant) with respect to quencher size, Calhoun et al.[9,15] have concluded that diffusion of acrylamide to W314 (4.5 Å from the surface) is slow even in the millisecond-to-second time scale of phosphorescence. These workers proposed that quenching of both fluorescence and phosphorescence of W314 by acrylamide is probably due to long-range electron exchange/transfer with Q in the aqueous phase.

Should acrylamide quench by physical contact with the chromophore and penetration be required for quenching the luminescence of internal Trp residues, then the rate constants ${}^{F}k_q$ and ${}^{P}k_q$ would necessarily have to be of a similar magnitude. Until now, only a few quenching experiments have been carried out utilizing both fluorescence and phosphorescence, and at present a comparison between ${}^{F}k_q$ and ${}^{P}k_q$ is possible only with ribonuclease (RNase) T_1 and W314 of LADH.[13] In either case, relative to solvent-exposed Trps, ${}^{F}k_q$ decreases much less than ${}^{P}k_q$, the large discrepancies raising serious concerns about the correct interpretation of ${}^{F}k_q$ or ${}^{P}k_q$ or both. As mentioned above, Calhoun et al.,[9] advocating a through-space quenching mechanism, have attributed the difference between ${}^{F}k_q$ and ${}^{P}k_q$ to a distinct distance dependence of the through-space quenching rate, $k(r)$. To the contrary, Ghiron et al.,[13] assuming collisional quenching by acrylamide penetration for fluorescence, have suggested that ${}^{P}k_q$ may not be a reliable parameter of the quencher−chromophore collisional frequency. Employing the triplet−triplet absorption technique, they observed solvent-dependent variations in the triplet quenching efficiency of acrylamide and from it inferred that, as opposed to ${}^{F}k_q$, ${}^{P}k_q$ may change considerably from one protein environment to another. Unfortunately, triplet−triplet absorption data may not be reliable because, under the conditions of these experiments, the triplet-state lifetime of free Trp is about 40-fold smaller than that measured by phosphorescence.[16]

Solvent-protected Trp residues exhibit room-temperature phosphorescence (RTP) lifetimes in the millisecond-to-second time range, and consequently, the delayed emission would be ideally suited for monitoring even extremely small bimolecular quenching rates.[8,13,15] For acrylamide quenching of Trp phosphorescence to be a useful monitor of molecular diffusion through proteins, however, long-range interactions must be negligible and ${}^{P}k_q$ needs to be proportional to the quenching−chromophore encounter frequency. The objective of this report is to critically assess the potential of ${}^{P}k_q$ as a probe of the dynamical structure of proteins. To this end, phosphorescence quenching experiments have been carried out to (1) evaluate the distance range of through-space interactions employing model indole compounds both in low-temperature rigid glasses and in fluid solutions; (2) determine the variability of the quenching efficiency in organic solvents; (3) evaluate ${}^{P}k_q$ for a series of proteins with increasing degree of Trp burial; and finally, (4) test for a possible correlation between ${}^{P}k_q$ and changes in structural flexibility as induced by metal binding to apoproteins, complex formation with coenzymes, and the addition of denaturing agents.

Materials and Methods

High-purity indole and *N*-acetyltryptophanamide (NATA) were purchased from Sigma (St. Louis, MO) and prior to use were recrystallized three times from ethanol/water. 2-(3-indoyl)ethyl phenyl ketone (IEPK), prepared according to the procedure of Tamaki,[21] was a gift from Dr. Lee, Department of Chemistry, Mc Gill University (Montreal, Canada). NAD^+, ADPR, and pyrazole were from Sigma (St. Louis, MO). Spectroscopic grade propylene glycol (PG) and dioxane were from Merck (Darmstadt) and prior to use were treated with reducing agent ($NaBH_4$) and distilled under vacuum. Ultrapure guanidine hydrochloride (GudnHCl) was from UBS Corp. (Cleveland, OH). Acrylamide (>99.9% electrophoresis purity) was from Bio-Rad Laboratories (Richmond, CA). The proteins horse liver alcohol dehydrogenase (LADH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from yeast were supplied by Boheringer (Mannheim, Germany). To remove NAD^+ from GAPDH, the enzyme was treated with activated charcoal as reported before.[17] Alkaline phosphatase (AP) from *Escherichia coli* and β-lactoglobulin A were obtained from Sigma. Ribonuclease (RNase) T_1 was purchased from Calbiochem Corp. (San Diego, CA). Copper-free azurin from *Pseudomonas aeruginosa* was a gift from Prof. Finazzi-Agrò, University of Roma (Tor Vergata, Italy). Water, doubly distilled over quartz, was purified by using a Milli-Q Plus system (Millipore Corp., Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt).

Sample Preparation for Phosphorescence Measurements. The complexes of LADH with ADPR and with NAD^+ plus pyrazole were formed simply by the addition of the reagents to a 2 μM protein solution to a final concentration of 430 μM for ADPR, 2 μM for NAD^+, and 10 mM for pyrazole. Cd-azurin was formed from apoazurin by the

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addition of $CdCl_2$ in a molar ratio of 2:1. Complex formation was verified by looking at the Trp fluorescence intensity in competition experiments with Cu^{2+}, as Cu-azurin is strongly quenched.[18] Within the first 2 h from the addition of Cu^{2+}, no change in fluorescence was observed in the Cd-azurin sample.

For measurements in low-temperature glasses, IEPK (3×10^{-3} M) and NATA (5×10^{-4} M) samples were equilibrated (2 h) under N_2 atmosphere in the dark and then placed in round spectrosil quartz cuvettes (4 mm i.d.). The steady-state emission intensities, upon excitation at 295 nm, were corrected for the absorption of acrylamide (A_{acr}^{295}) at that wavelength by the factor $-\log(A_{acr}^{295}/2)$.[19] For phosphorescence lifetime measurements in fluid solutions at ambient temperature, the samples were placed in 5×5 mm^2 quartz cuvettes especially designed for allowing a thorough removal of O_2 by the alternating application of moderate vacuum and inlet of ultrapure N_2.[16] In all experiments, the concentration of NATA and indole was 5 μM, and that of proteins ranged from 1 to 5 μM.

Luminescence Measurements. Routine fluorescence spectra were obtained with a Jasco FP-770 fluorometer. A conventional homemade instrument was employed for all phosphorescence intensity, spectra, and lifetime measurements in low-temperature glasses.[20] The excitation provided by a Cermax xenon lamp (LX 150 UV; ILC Technology, Sunnyvale, CA) was selected by using a 0.25-m grating monochromator (Jobin-Yvon, H25), and the emission dispersed by a 0.25-m grating monochromator (Jobin-Yvon, H25) was detected with an EMI 9635 QB photomultiplier. Phosphorescence decays in fluid room-temperature solutions were measured on an apparatus described before.[16] Briefly, pulsed excitation was provided by a frequency-doubled flash-pumped dye laser (UV 500 M-Candela) ($\lambda_{ex} = 292$ nm) with a pulse duration of 1 μs and an energy per pulse of typically 1−10 mJ. The emitted light was collected at 90° from the excitation direction and selected by a filter combination with a transmission window between 420 and 460 nm. The photomultipliers were protected from the intense excitation and fluorescence light pulse by a high-speed chopper blade that closed the slits during laser excitation. The minimum dead time of the apparatus is about 10 μs. All the decaying signals were digitized and averaged by a computerscope system (EGAA; RC Electronics). Subsequent analysis of decay curves in terms of discrete exponential components was carried out by a nonlinear least-squares fitting algorithm, implemented by the program Global Analysis. All reported decay data are averages of three or more independent measurements. The reproducibility of τ_p was typically better than 5%.

The viscosity of PG and of dioxane was determined with an Ostwald viscosimeter calibrated with a glycerol/water (50/50, w/w) solution of known viscosity.

Results

Phosphorescence Quenching of IEPK by Acrylamide in a Glass Matrix. Acrylamide quenches both fluorescence and phosphorescence of indole derivatives. To study selectively the interaction between acrylamide and the excited triplet state of indole, quenching of the precursor fluorescent state must be avoided. For this, an indole derivative that exhibits only phosphorescence emission was employed. This is accomplished with IEPK, a conjugated indole compound with an effective intersystem crossing quantum yield of 1. Thanks to the lower energy of the excited singlet state of the acetophenone moiety covalently bound to indole, the fluorescence from the latter is totally quenched by intramolecular indole to acetophenone singlet−singlet energy transfer. Following intersystem crossing in acetophenone ($\Phi_{isc} = 1$), the excitation is back-transferred to the lower-lying triplet state of indole. Thus, at any excitation wavelength, the emission from IEPK is represented entirely by indole phosphorescence.[21]

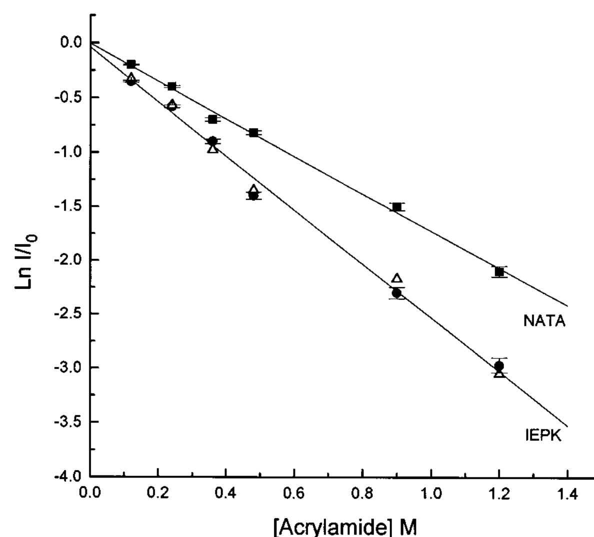

Figure 1. Relative fluorescence yield of NATA (■) and phosphorescence yield of IEPK (●) as a function of acrylamide concentration in a PG/phosphate buffer (20 mM, pH 7)/methanol (50/45/5, v/v/v) glass at 120 K. The phosphorescence yield of IEPK calculated from the area under the decay curves ($t \geq 2$ ms) in time-dependent experiments is also indicated (△). The concentration of NATA was 5×10^{-4} M, and that of IEPK was 3×10^{-3} M. The intensity values are averages of three separate determinations; error bars indicate the range.

The phosphorescence emission ($\lambda_{ex} = 295$ nm) of IEPK (3×10^{-3} M) was measured in a PG/phosphate buffer (20 mM, pH 7)/methanol (50/45/5, v/v/v) glass at 120 K as a function of acrylamide concentration. The interaction with acrylamide leads to quenching of the phosphorescence intensity and to a reduction of the triplet lifetime. The relative steady-state phosphorescence intensity of IEPK, $P(c)/P(0)$, at various acrylamide concentrations is shown in Figure 1. Before interpreting the decrease in $P(c)/P(0)$ in terms of a long-distance indole−acrylamide interaction, we point out that quenching can, in principle, result from the formation of dark ground-state complexes. Under the present experimental conditions, there is no evidence to indicate complex formation. At 1.2 M acrylamide, the highest concentration employed, both the absorption (at ambient temperature) and the phosphorescence spectrum (at 120 K) are not affected by the quencher, an indication that strong ground-state or excited-state chromophore−quencher complexes are not formed. Furthermore, whereas an exponential dependence of $P(c)/P(0)$, as found here (Figure 1), is consistent with a random distribution of chromophore−quencher distances/orientations, static quenching by nonfluorescent complexes is predicted to follow Stern−Volmer concentration dependence.[22]

We shall assume the indole−acrylamide distance distribution to be completely random and interpret the decrease in phosphorescence yield in terms of Perrin's active sphere model,[22,23]

$$P(\mathrm{c})/P(0) = \exp(-cv) \tag{1}$$

$$v = (4\pi/3)(r_q^{\,3} - r_0^{\,3}) \tag{2}$$

where c is the quencher concentration (number/volume), v is the volume of the active sphere, $r_0 = (\bar{r}_{indole} + \bar{r}_{acrylamide})$ is the sum of the average molecular radii, and r_q is the average center-

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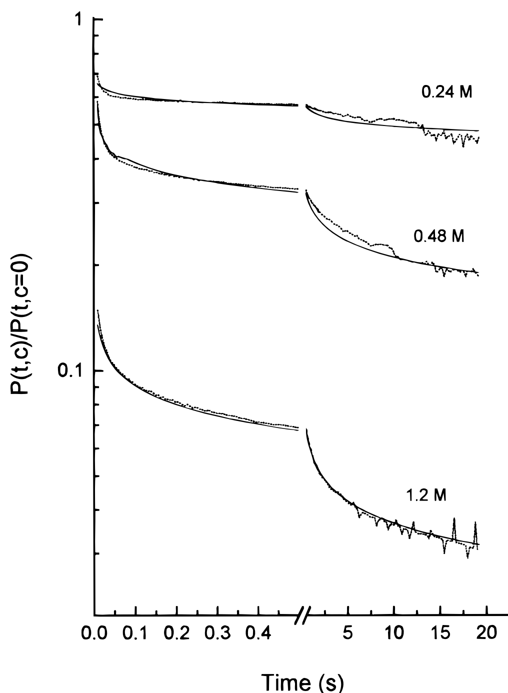

Figure 2. Decay of IEPK phosphorescence ($\lambda_{ex} = 292$ nm, $\lambda_{em} = 440$ nm) in a glass at 120 K at some representative concentrations, *c*, of acrylamide. All intensities $P(t,c)$ are normalized by the control sample $P(t,0)$. Full lines represent the curves obtained from the best fit of the data by eq 4. Other experimental conditions are as in Figure 1.

to-center distance at which the rate of quenching is equal to the unperturbed phosphorescence lifetime, τ_0. When r_0 is set at 4 Å,[14] the slope of the semilogarithmic plot yields $r_q = 9.9$ Å.

For comparison, Figure 1 also reports the decrease in steady-state fluorescence intensity, $F(c)/F(0)$, of NATA (5×10^{-4} M) under the same experimental conditions. Again, the $F(c)/F(0)$ ratio obeys an exponential dependence on acrylamide concentration. When this is analyzed in terms of eqs 1 and 2, it yields an interaction radius $r_q = 8.74$ Å. If r_0 is set at 5 Å, a magnitude that was found to better fit the quenched fluorescence decay of NATA in PG at 210 K,[14] then r_q is 9.0 Å in fair agreement with 9.4 Å estimated in that study.

Upon pulsed laser excitation at 292 nm, the phosphorescence of IEPK in the PG/buffer glass decays in an approximately exponential fashion, and except for a 4−5% short-lived contribution from solvent impurities during the first 0.5 s, the lifetime, τ_0, is about 5 s. In the presence of acrylamide, the solvent-corrected decays are no longer exponential, and at larger quencher concentrations, more shorter-lived components contribute to the emission. Representative decays ($\lambda_{em} = 440$ nm), normalized by the intensity of IEPK control, $P(t,c)/P(t,0)$, are shown in Figure 2.

It should be noted that the shortening of the triplet lifetime accounts completely for the quenching of the steady-state intensity because the areas under the decay curves, as seen in Figure 1, follow closely the decrease in phosphorescence yield determined in steady-state measurements. This again confirms that quenching by dark complexes is negligible.

In rigid media, lifetime shortening and heterogeneous decays are consistent with quenching interactions that go beyond van der Waals contact and with a distribution of quencher−chromophore geometrical arrangements. The decays $P(t,c)/P(t,0)$ were fitted assuming an exponential distance dependence of the quenching rate, $k(r)$, a dependence typical of both of electron-transfer reactions[24] and electron exchange interactions.[25,26] Namely,

$$k(r) = k_0 \exp[-(r - r_0)/r_e] \quad (3)$$

where k_0 is the maximum rate at the contact distance r_0 and r_e is the attenuation length. For a random distribution of chromophore−quencher distances, and assuming that the strong dependence on separation determines the distribution of rates (i.e., orientation effects neglected), Huddleston and Miller[27] derived an expression that is applicable to the decay of phosphorescence, in the form

$$P(t,c)/P(t,0) = \exp\{-(4\pi/3)c[(r_0 + r_e \ln(gk_0t))^3 - r_0^3]\} \quad (4)$$

where g is a numerical factor equal to 1.9. The above expression gave reasonably good fits (combined $\chi^2 \leq 3.5$) of phosphorescence decay data (Figure 2). Setting $r_0 = 4$ Å, the best-fit values of the parameters are $k_0 = 1.2 \times 10^8$ s^{-1} and $r_e = 0.29$ Å. With this distance dependence of the rate, $k(r_q) = 1/\tau_0 = 0.2$ s^{-1} predicts an average transfer radius of 9.8 Å, in good agreement with $r_q = 9.9$ Å obtained from steady-state measurements. It is also interesting to note that, in comparison with the parameters $k_0 = 6 \times 10^{13}$ s^{-1} and $r_e = 0.32$ Å obtained from fitting fluorescence decay data,[14] the main difference with phosphorescence quenching lies in an about 5 orders of magnitude smaller preexponential term.

Quenching of NATA Phosphorescence by Acrylamide in Liquids. The effectiveness of acrylamide as a quencher of indole phosphorescence in aqueous solutions (10 mM phosphate buffer, pH 7) at 20 °C was investigated with the Trp derivative NATA. The addition of acrylamide to a solution of NATA (5×10^{-6} M) was found to shorten its phosphorescence lifetime but to maintain the decay monoexponential throughout. The decrease in τ as a function of acrylamide concentration (Figure 3) yields a linear Stern−Volmer plot, from which a bimolecular quenching rate constant ${}^{P}k_q = (1.5 \pm 0.1) \times 10^9$ M^{-1} s^{-1} is obtained. This value is only slightly smaller than 2×10^9 M^{-1} s^{-1} determined by Ghiron et al.[13] using the flash photolysis technique. Such magnitude of k_q is one-fifth of the estimated quencher−chromophore diffusion-limited rate constant (7.5×10^9 M^{-1} s^{-1})[4] and, therefore, indicates an encounter quenching efficiency $\gamma = 0.2$. Values of γ between 0.5 and 0.1 are typical of triplet quenching processes and are generally attributed to the $^3/_5$−$^1/_9$ spin statistical factor.[28,29]

To test for a possible influence of the nature of the solvent on γ, a range of organic solvents varying in dielectric constant and proticity were examined. Unfortunately, among those available of spectroscopic grade acetonitrile, formamide, *N*-methylformamide, ethanol, ethylene glycol, glycerol, dioxane, and PG, only the latter two were sufficiently free of quenching impurities to yield the unperturbed (1 ms) lifetime of NATA. The effects of acrylamide on the lifetime of indole in dioxane and of NATA in PG at 20 °C are displayed in Figure 3. From the gradient of the linear Stern−Volmer plots, k_q is determined to be 4.3×10^7 M^{-1} s^{-1} in PG and 1.6×10^8 M^{-1} s^{-1} in dioxane. The latter value is a factor of 2 smaller than that

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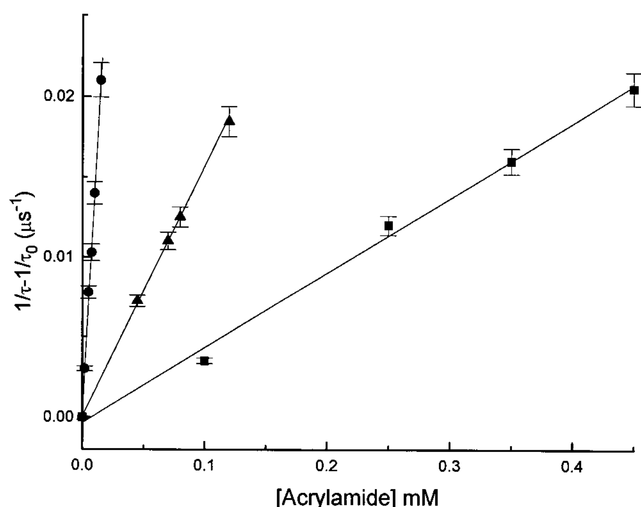

Figure 3. Lifetime Stern−Volmer plots for the quenching of NATA phosphorescence by acrylamide in phosphate buffer (20 mM, pH 7) (●), in PG (▲), and for the quenching of indole phosphorescence in dioxane (■). The chromophore concentration is 5×10^{-6} M. The temperature is 20 °C. The values reported are averages from three separate experiments, and the error bars indicate the range. The unperturbed lifetime τ_0 is about 1 ms in buffer and dioxane and is 1.3 ms in PG.

Table 1. Acrylamide Phosphorescence Quenching of Indole Derivatives in Various Solvents at 20 °C

chromophore	solvent	ϵ^a	η (cP)	${}^{P}k_q$ (M^{-1} s^{-1})	$\gamma = {}^{P}k_q/k_d$
NATA	H_2O	78.4	1.0	1.5×10^9	0.2
NATA	PG	19	62.1	4.3×10^7	0.35
indole	dioxane	2.2	1.2	1.6×10^8	0.025

[a] ϵ is the dielectric constant at 25 °C taken from Riddick, J. A., Bunger, W. B., Eds. *Organic Solvents*; Techniques of Chemistry, II; Wiley-Interscience: New York, 1970.

determined by flash photolysis.[13] When the bimolecular quenching rate constants are normalized by the respective viscosity of the solvent (Table 1), one obtains that the quenching efficiency for NATA is similar in water and in PG but is almost 10-fold smaller for indole in dioxane.

Quenching of Trp Phosphorescence in Proteins by Acrylamide. Protein systems and experimental conditions were selected in order to test for possible correlations existing between quenchability by acrylamide and structural features such as the surface proximity of the indole side chain and the flexibility of the Trp site, as inferred from the magnitude of the intrinsic phosphorescence lifetime, τ_0.[16] The proteins examined, their τ_0 at 20 °C, and the thickness of the protein spacer separating the indole ring from the aqueous phase, r_p, are listed in Table 2.

The RTP emissions of some proteins, namely LADH, GAPDH, and β-lactoglobulin, are intrinsically heterogeneous and were found to remain so even when the average phosphorescence lifetime is considerably reduced by acrylamide. Because in each protein the RTP is due to a single Trp residue per subunit, such heterogeneity reflects the presence of more then one stable conformation of the macromolecule,[16,30] each with its distinct τ_0 and acrylamide quenching rate constant. For convenience, and also because an evaluation of individual quenching rates is model dependent, lifetime Stern−Volmer plots were all constructed from the average lifetime, $\tau_{av} = \Sigma\alpha_i\tau_i$, obtained in general from a biexponential fitting of phosphorescence decays. Thus, the value of ${}^{P}k_q$ derived from the gradient of these plots is an averaged quantity.

Representative lifetime Stern−Volmer plots are shown for LADH and azurin in Figure 4. The data with LADH (Figure 4a) emphasize the strong modulation of the acrylamide quenching effectiveness by experimental conditions that change the flexibility of the polypeptide without altering the overall topology of the native protein fold. In fact, in 0.5 M GudnHCl, both fluorescence and circular dichroism spectra are native-like, and the protein still exhibits catalytic activity, although at a reduced rate relative to that of buffer.[31] However, with the addition of GudnHCl, τ_0 decreases from 430 to 186 ms, and the greater mobility of the structure about W314 inferred from the change in τ_0 is accompanied by a 3-fold increase in ${}^{P}k_q$. Further, complex formation with ADPR or with NAD^+ plus pyrazole increases τ_0 from 430 ms to about 1 s.[32] In this case, the more rigid structure about W314 is reflected in a 10-fold reduction of ${}^{P}k_q$. Note that the modulation of structural flexibility in LADH reported by τ_0 is totally consistent with thermal stability data.

The same point is also illustrated by Cd^{2+} binding to apoazurin, although the Stern−Volmer plot of the metalated protein is not linear (Figure 4b). Data at low acrylamide concentrations, below 0.23 M, show that, upon metal binding, ${}^{P}k_q$ is reduced by over 35-fold. Although τ_0 does not increase appreciably on going from apo- to holostructure, the latter is presumably much more rigid and compact because its thermal unfolding temperature increases from 62 to 93 °C.[33] Above 0.23 M acrylamide, the Stern−Volmer plot of Cd-azurin exhibits an unusual saturation effect as τ remains practically constant up to the highest (1.5 M) concentration examined. Such behavior was found to be quite reproducible. Possible explanations for it are advanced in the Discussion section.

Among the proteins of Table 2, ${}^{P}k_q$ has been previously determined for RNase T_1 employing the triplet−triplet absorption method[13] and for LADH using phosphorescence quenching.[15] Compared to these previous reports, the present value of ${}^{P}k_q$ is about 2-fold smaller for LADH and 4-fold smaller for RNase T_1.

Inspection of Table 2 shows a wide variability in the quenching rate constant among the protein examined, ${}^{P}k_q$ decreasing from 5×10^4 M^{-1} s^{-1} for the most superficial Trp residue of RNase T_1 to 10^{-1} M^{-1} s^{-1} for W109 of AP, which is the most deeply buried of the entire set. For the latter, molar quencher concentrations are needed to affect the phosphorescence lifetime. Given the propensity of acrylamide to form weak associations with proteins, one cannot exclude that part of the reduction in RTP lifetime of AP may be caused by acrylamide binding.

Discussion

This study establishes that, in the millisecond-to-second time scale of phosphorescence, acrylamide can quench the emission of even highly buried, inaccessible Trp residues in proteins. Although, in principle, the process could involve either long-range interactions with the quencher in the solvent or its hindered diffusion through the globular structure, experimental evidence shows that, in general, the former route makes negligible contributions.

Through-Space Quenching of Trp Phosphorescence by Acrylamide. The relevance of through-space interactions can

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Table 2. Acrylamide Bimolecular Phosphorescence Quenching Rate Constant for NATA and for Internal Trp Residues in Proteins, at 20 °C (Theoretical Estimates of the Through-Space Rate, $^{P}k_q(r_p)$, Based on $k(r)$ and Crystallographic Data (r_p), Are Also Included)

sample	buffer[a]/pH	$[Acr]_{max}$ (mM)	Trp no.	$\bar{\tau}_0$ (ms)	$^{P}k_q$ ($M^{-1}\,s^{-1}$)	r_p (Å)	$^{P}k_q(r_p)$ ($M^{-1}\,s^{-1}$)	$\eta(^{P}kq)$[c] (cP)	$\eta(^{P}k_q)/\eta(\tau_0)$[d]
NATA	Phos/7.5	1.48×10^{-2}		1	1.5×10^{9}				
RNase T_1	NaAc/5.5	5	59	31	5.9×10^{4}	2	1.5×10^{4}	1.3×10^{5}	
LADH	Phos/7.5	1	314	430	1.2×10^{4} [b]	4.5	3.8	6.3×10^{5}	3.2×10^{1}
LADH + 0.5 M GudnHCl	Phos/7.5	0.3	314	186	3.3×10^{4} [b]	4.5	3.8	2.3×10^{5}	4.7×10^{1}
LADH−NAD^+-Pyr	Phos/7.5	40	314	1020	9.5×10^{2} [b]	4.5	3.8	7.9×10^{6}	5.0×10^{1}
LADH-ADPR	Phos/7.5	40	314	1125	1.1×10^{3}	4.5	3.8	6.5×10^{6}	4.1×10^{1}
asparaginase	Tris/7.5	1700	66	3.5	5.3×10^{1}	5	0.7	1.4×10^{8}	5×10^{6}
GAPDH	Tris/7.5	100	84	160	3.4×10^{2} [b]	5.5	0.13	2.2×10^{7}	0.6×10^{4}
β-lactoglobulin	NaAc/5.5	5	19	48	4.2×10^{4} [b]	6	2.4×10^{-2}	1.8×10^{5}	0.2×10^{4}
apoazurin	Tris/7.5	1000	48	600	3.2×10^{1}	8	2.9×10^{-5}	2.3×10^{8}	0.5×10^{4}
Cd-azurin	Tris/7.5	1500	48	540	8.6×10^{-1}	8	2.9×10^{-5}	8.7×10^{9}	23.9×10^{4}
AP	Tris/7.5	1700	109	2060	1×10^{-1}	11	1.1×10^{-9}	7.5×10^{10}	10.0×10^{4}

[a] Phos is 20 mM potassium phosphate; NaAc is 10 mM sodium acetate; Tris is 50 mM Tris-HCl. [b] Average $^{P}k_q$ obtained from $(1/\bar{\tau}) - (1/\bar{\tau}_0)$. [c] $\eta(^{P}k_q) = 7.5 \times 10^{9}/^{P}k_q$. [d] $\eta(\tau_0)$ was calculated from the empirical relationship between τ_0 and η reported by Strambini and Gonnelli.[16]

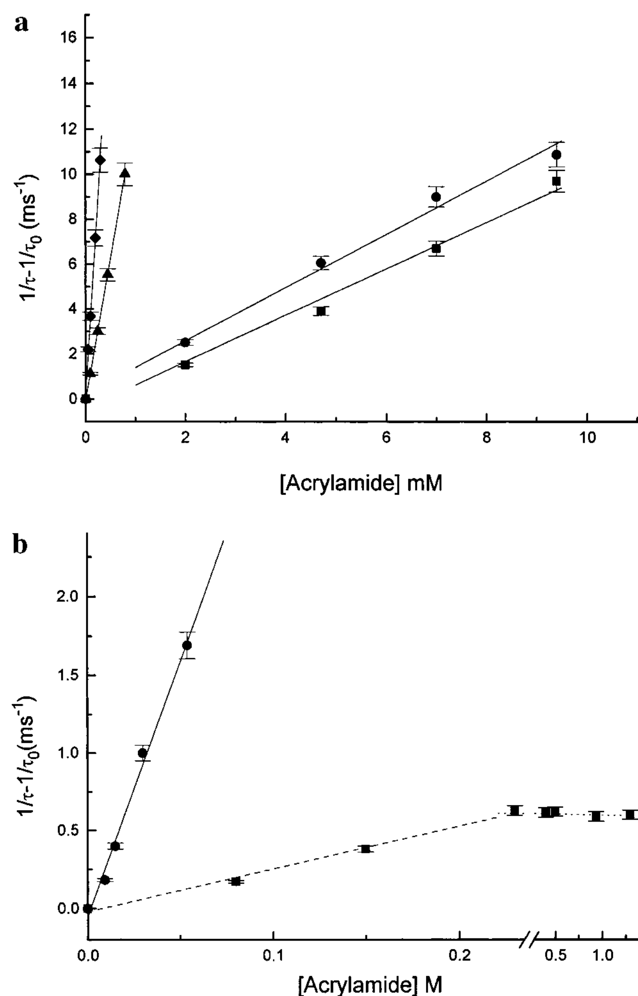

Figure 4. Examples of lifetime Stern−Volmer plots for the quenching of Trp phosphorescence in proteins by acrylamide. (a) W314 of LADH (in 20 mM phosphate buffer, pH 7.5) upon varying experimental conditions: (▲) LADH; (◆) LADH + 0.5 M GudnHCl; (●) LADH−ADPR binary complex; (■) LADH−NAD^+−pyr ternary complex. (b) Apoazurin (●) and Cd-azurin (■) in Tris-HCl 50 mM, pH 7.5. The temperature is 20 °C.

be assessed from $k(r)$, the distance dependence of the reaction determined with model indole compounds in glasses and in liquid solutions. The process presumably involves an electron exchange or transfer,[9,14] and the expected exponential dependence of $k(r)$[24,25] was, indeed, found to fit nicely the acrylamide-perturbed phosphorescence decays in rigid matrixes. Considering a static, random distribution of acrylamide molecules, the fitting parameters yielded an average interaction radius of 10 Å and a rate $k(r) = 1.2 \times 10^{8} \exp[-(r - 4)/0.29]\ s^{-1}$. These results confirm that the through-space interactions between the triplet state and acrylamide do occur, although the small magnitude of both the contact rate (k_0) and the attenuation length implies that they are short-range. Preexponential terms much smaller than $10^{13}\ s^{-1}$, the maximum value for electron-transfer reactions, and attenuation lengths shorter than 1−1.5 Å have also been observed for electron transfer between the triplet state of indole and Cu^{2+} [34] or to other redox agents.[35]

The above estimate of $k(r)$ refers to a rigid matrix at 120 K. According to Marcus theory,[24] electron-transfer rates can be affected by both temperature and solvent relaxation, their effects being predominant on the preexponential term, k_0. The variation of k_0 from low-temperature glasses to liquids at ambient temperature can, in some cases, be estimated from the bimolecular quenching rate constant in these media. Under conditions for which quenching in solution is less than diffusion control, namely when $k_q < k_d$, as is the case with phosphorescence, k_q is simply related to k_0 by the expression[36]

$$k_q^{-1} = (0.287 k_0)^{-1} + k_d^{-1} \quad (5)$$

From $k_q = 1.5 \times 10^{9}\ M^{-1}\ s^{-1}$ for the quenching of NATA phosphorescence in aqueous solutions at 20 °C and $k_d = 7.5 \times 10^{9}\ M^{-1}\ s^{-1}$,[4] one derives $k_0 = 6.5 \times 10^{9}\ s^{-1}$. With this correction, the distance dependence of the quenching reaction in water at ambient temperature becomes $k(r) = 6.5 \times 10^{9} \exp[-(r - 4)0.29]\ s^{-1}$.

Here, k_0 was derived by taking water as a solvent, where the quenching efficiency in an indole−acrylamide collision, $\gamma = k_q/k_d$, is 0.2. A similar efficiency, $\gamma = 0.35$, is found in PG, whereas γ is 10-fold less in dioxane, the only other organic solvent in which it could be measured. According to eq 5, in dioxane k_0 is 10 times smaller than that in water. Note that for an electron-transfer reaction solvent effects on k_0 are to be expected, as this parameter is related to the overall hexothermicity of the reaction and solvent relaxation about the reactants makes a nonnegligible contribution to it.[24] The energy of solvent relaxation depends on the dielectric constant (ϵ) and on H-bond formation. Because among organic solvents dioxane

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is aprotic and possesses one of the smallest dielectric constant ($\epsilon = 2$) the solvent relaxation energy, and consequently k_0, are bound to represent lower limits within the range of possible values. A variability of γ by a factor of 15 was reported for a larger number of solvents by flash photolysis.[13] The sensitivity of $k(r)$ to the nature of the medium points out that in proteins the rate of through-space quenching and the quenching efficiency in a collisional encounter will vary with the Trp microenvironment. However, since relative to water and dioxane protein environments are intermediate in polarity and H-bonding capacity, the variability of both $k(r)$ and γ is expected not to exceed 10-fold.

As opposed to phosphorescence, for fluorescence quenching $\gamma = 1$ in a variety of solvents tested.[4] The reason for it is now evident because, for fluorescence quenching, k_0 largely exceeds k_d ($k_0 = 6 \times 10^{13}$ s^{-1} in PG at 293 K),[14] and according to eq 5 $k_q \approx k_d$ in any solvent. Of course, $\gamma = 1$ for the free chromophore does not mean that the quenching rate, ${}^{F}k(r)$, will be medium independent. Environmental effects in proteins, for example, may play an important role when quenching through-space across an impermeable protein spacer becomes the dominant pathway.

Knowledge of $k(r)$ is useful for estimating the magnitude of through-space interactions between Trp residues in proteins and acrylamide molecules that are either freely diffusing in the aqueous phase or bound to the macromolecule. For the typical $1-10^3$-ms range of RTP lifetimes, we note that static quenching extends over distances of 4−6 Å beyond van der Waals contact. Hence, binding of acrylamide to even superficial sites can seriously affect the lifetime of internal Trp residues and mislead the interpretation of quenching data in terms of quencher mobility through the protein matrix.

Dynamic quenching of a protected Trp residue by acrylamide in the solvent can be readily calculated from $k(r)$ and the minimum thickness of the protein spacer, r_p. For a protein model with an effective planar surface, the bimolecular rate constant ${}^{P}k_q(r_p)$ for dynamic quenching in the rapid diffusion limit, which certainly applies to long-lived ($\tau \geq 1$ ms) emission, is given by[35]

$$ {}^{P}k_q(r_p) = 2\pi(10^{-3})Nk_0[r_p\, r_e^{\,2} + 2r_e^{\,3}] \exp(-r_p/r_e)\ \mathrm{M^{-1}\ s^{-1}} \quad (6) $$

where N is Avogadro's number and r_e is the attenuation length (units of r_e and r_p in cm). For each protein examined in this study, knowledge of the phosphorescing Trp[37] and its location from crystallographic data permits the estimation of r_p and of ${}^{P}k_q(r_p)$. Theoretical estimates of ${}^{P}k_q(r_p)$ based on $k_0 = 6.5 \times 10^9$ s^{-1} and $r_e = 0.29$ Å are reported in Table 2. The comparison with experimental values of ${}^{P}k_q$ (Figure 5) emphasizes that only for the most superficial ($r_p \approx 2$ Å) W59 of RNase T_1 can quenching from the solvent make a significant contribution. For the rest, ${}^{P}k_q(r_p)$ is 3−10 orders of magnitude smaller than ${}^{P}k_q$, demonstrating that for $r_p \geq 4$ Å through-space interactions play no role in quenching these internal Trp residues. This conclusion does not support the hypothesis that phosphorescence quenching of LADH by acrylamide involves long-range interactions with the quencher in the solvent.[15]

Quenching Mechanisms and Interpretation of k_q in Terms of Structural Fluctuations. Besides long-range interactions, two other mechanisms have been proposed to account for protein fluorescence and phosphorescence quenching by added solutes.

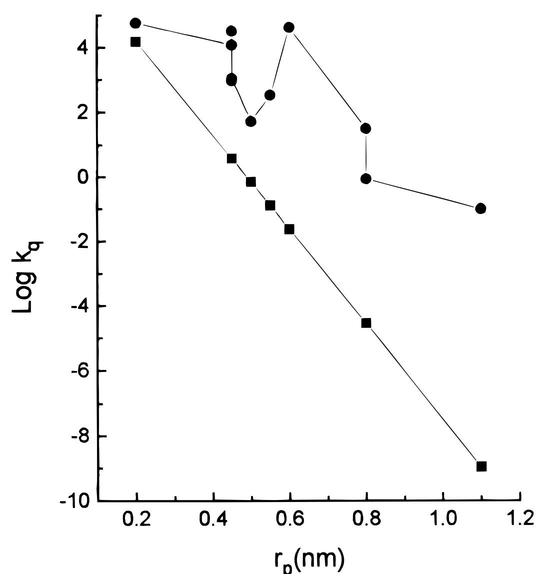

Figure 5. Comparison between experimental (●) and theoretical (■) acrylamide bimolecular quenching rate constants (see Table 2) as a function of the distance between the triplet probe and the protein surface.

These are (i) transient local unfolding or large amplitude structural fluctuations of the polypeptide that bring the normally buried chromophore in proximity with the solvent and (ii) migration of the quencher through the protein matrix to within interaction distance of the indole ring.

The former is sometimes referred to as the gating mechanism in that the rate, k_q[Q], is limited by the frequency of the putative conformational transition, ν_g.[38] In this model, $k_q[Q] \leq \nu_g$, and saturation effects are expected to occur at sufficiently large [Q]. Except for Cd-azurin, for the other proteins the lifetime Stern−Volmer plots were linear in the range of acrylamide concentrations employed in this study. With Cd-azurin, the quenching rate reached a maximum value of 0.6 s^{-1} in 0.23 M acrylamide and remained constant up to 1.5 M (Figure 4b). It is premature to say whether a gating mechanism or other processes such as acrylamide binding are responsible for this singular behavior. For the other proteins of Table 2, gating cannot be excluded but appears unlikely. For these ${}^{P}k_q[Q]_{max}$ ranges between 10 and 100 s^{-1} (Table 2), and therefore their ν_g would have to be larger. However, the exposure of such deeply buried Trp residues would require drastic rearrangements of the globular structure, and these would be characterized by activation barriers and rates presumably not too different from those normally observed for the unfolding of small proteins (rates $<10^{-1}$ s^{-1}). Furthermore, it should also be noted that structural fluctuations of such magnitude and frequency are not compatible with the long-lived and sometime heterogeneous phosphorescence decay of these proteins. Because Trp residues on the protein surface invariably exhibit submillisecond lifetimes, even transient exposure of buried chromophores would deactivate the triplet state and cause the intrinsic lifetime to become $\tau_0 \leq 1/\nu_g$.

Whether or not gating makes nonnegligible contributions to the quenchability of the proteins examined, important indicators of structural flexibility emphasize that the variability of ${}^{P}k_q$ in Table 2 is clearly correlated to the dynamical structure of the macromolecule. One such indirect indicator is the thermal stability through the natural link between stability and compactness of the globular structure. Binding of Cd^{2+} to apoazurin, which raises its melting temperature, T_m, by 31 °C,[33] decreases

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$^{P}k_q$ by at least 35-fold. Likewise, modulation of the thermal stability of LADH by GudnHCl, which reduces it, and complex formation with NAD^+ or the ADPR analogue, which enhances it,[39] results in a 3-fold increase and a 10-fold decrease of $^{P}k_q$, respectively.

Another important monitor of structural flexibility is the intrinsic phosphorescence lifetime, τ_0. The empirical relationship between τ_0 and the medium viscosity[16] can provide a direct measure of the polypeptide fluidity in the region about the triplet probe. Naturally, a close correspondence between the two "microviscosities", one derived from the magnitude of τ_0, $\eta(\tau_0)$, and the other from $^{P}k_q$ by assuming that it represents hindered acrylamide diffusion through the protein matrix, $\eta(^{P}k_q)$, would be anticipated unless both $^{P}k_q$ and τ_0 were influenced by the same kind of segmental motions. Large discrepancies between $\eta(\tau_0)$ and $\eta(^{P}k_q)$ are therefore inevitable whenever τ_0 is unduly short owing to a local quenching reaction or when acrylamide diffusion is not limited by structural fluctuations of the inner core of the structure hosting the chromophore. In Table 2, the ratio $\eta(^{P}k_q)/\eta(\tau_0)$, is shown to range from 32 for LADH to 5×10^6 for asparaginase. The first observation is that the apparent frictional drag governing acrylamide diffusion is always much greater than that anticipated on the basis of the "local viscosity". This suggests that few of the structural fluctuations that confer flexibility to the Trp sites are sufficiently large in amplitude to permit diffusional jumps to acrylamide. The second observation is that, if exception is made for the lower and upper limits of LADH and asparaginase, respectively, then the ratio $\eta(^{P}k_q)/\eta(\tau_0)$ is, within 1 order of magnitude of 10^4, roughly constant for a 10^6 variation in $^{P}k_q$. In consideration of the complete disregard for individual details of secondary and tertiary structure and of the many possibilities for discordant estimates of the "internal viscosity" by τ_0 and $^{P}k_q$, such correlation between two totally independent parameters is remarkable. Note that this correlation between τ_0 and $^{P}k_q$ is even better when the comparison of ratios is limited to the same macromolecule. For LADH, $\eta(^{P}k_q)/\eta(\tau_0)$ is practically constant over the 30-fold change in $^{P}k_q$, observed on going from 0.5 M GudnHCl to the coenzyme complexes.

The outer values of the viscosities ratio for LADH and asparaginase may have simple explanations. For LADH, $^{P}k_q$ seems unduly large for the local rigidity of the site (τ_0) and suggests that acrylamide diffusion to the proximity of W314 is greatly facilitated. Because W314 is the only residue that lies at the subunit interface of the dimeric molecule, acrylamide permeation may be facilitated by transient partial subunit dissociation. For asparaginase, a small $^{P}k_q$ and small crystallographic *B*-factors[40] in the region of W66 are both indications of a rigid local structure, and therefore a τ_0 of 3.5 ms appears unduly short. In a previous report,[5] τ_0 was found to be 50 ms, and it is therefore possible that, in our protein stock, τ_0 may be affected by an intramolecular quenching reaction. Adopting 50 ms for τ_0, the ratio $\eta(^{P}k_q)/\eta(\tau_0)$ becomes 5×10^4, in line with the rest.

Disparity in k_q between Fluorescence and Phosphorescence Quenching. Until the work of Lakowicz et al.[14] on the distance dependence of the quenching interaction, it was generally assumed that Trp fluorescence quenching by acrylamide involved a direct collision or a very close encounter with the chromophore. This presumed requirement, together with a peculiar dependence of $^{F}k_q$ of internal Trps on temperature, pressure, solvent viscosity (η_s), and quencher size, led many workers to conclude that acrylamide can migrate through proteins on the nanosecond time scale.[4] Accordingly, any reduction in $^{F}k_q$, relative to $(3-4) \times 10^9$ M^{-1} s^{-1} for solvent exposed Trps, has been interpreted in terms of hindered penetration of acrylamide and, therefore, as a measure of the dynamics of protein structures. This picture is not supported by the present phosphorescence study, as the fall from 5 to 10 orders of magnitude in $^{P}k_q$ (Table 2) demonstrates unequivocally that acrylamide diffusion through globular proteins is drastically slowed and that in the nanosecond time scale these macromolecules are effectively impermeable to the quencher.

This point is also emphasized by the disparity between $^{F}k_q$ and $^{P}k_q$ on the same macromolecule. Among the proteins of Table 2, $^{F}k_q$ has been reported for RNase T_1 (2×10^8 M^{-1} s^{-1}) and W314 of LADH (10^7 M^{-1} s^{-1}).[13] Relative to the free chromophore, the reduction in $^{F}k_q$ is 35-fold for RNase T_1 and 650-fold for LADH, whereas the corresponding reductions in $^{P}k_q$ are 2.4×10^4- and 1.5×10^5-fold, respectively. The discrepancy between the two methods, even allowing for 10-fold reduction in γ on changing from an aqueous to a protein environment, still amounts to about 2 orders of magnitude. Disagreement between fluorescence and phosphorescence quenching has been pointed out before, and various hypotheses have been put forth to account for it. Calhoun et al.,[9,15] arguing that acrylamide is too large a molecule to penetrate the protein matrix, proposed that quenching occurred from outside, through either long-range interactions or transient exposure of the chromophore. In this case, the disparity between $^{F}k_q$ and $^{P}k_q$ has been attributed to possible differences in mechanism or distance dependence of the interaction. On the other hand, using triplet−triplet absorption data, Ghiron et al.[13] found a solvent dependency of the triplet quenching efficiency and proposed that $^{P}k_q$ may vary significantly with the protein environment.

Here, we propose an alternative interpretation of fluorescence quenching data[5] and suggest that the discrepancy between $^{F}k_q$ and $^{P}k_q$ originates mainly from the large difference between $^{F}k(r)$ and $^{P}k(r)$. According to the rate of through-space fluorescence quenching, $^{F}k(r) = 6 \times 10^{13} \exp[-(r-5)/0.32]$,[14] the critical distance is around 9 Å, center to center. Therefore, superficial Trps ($r_p \leq 4$ Å) are quenched effectively through-space, and for proteins such as RNase T_1 ($r_p = 2$ Å) acrylamide quenching can be completely from the outside. For these Trps, a magnitude of $^{F}k_q < k_d$ may reflect essentially the distance dependence of the quenching reaction whose rate is given by

$$^{F}k_q \approx k_d\, ^{F}k(r_p)/(^{F}k(r_p) + k_{-d} + 1/\tau_F) = k_d\, ^{F}\gamma \qquad (7)$$

where k_{-d} is the rate at which acrylamide diffuses away from the protein surface, τ_F is the unperturbed fluorescence lifetime, and $^{F}\gamma$ is the probability that an encounter of acrylamide with the protein surface leads to fluorescence quenching. The near equality sign takes into account the fact that only a fraction of the protein surface is in proximity (within r_p) of the chromophore. According to eq 7, the dependence of $^{F}k_q$ on temperature, pressure, solvent viscosity (η_s), and quencher type should be sought on the differential effects of these parameters on $^{F}k(r_p)$ and k_d. Thus, the application of high hydrostatic pressure having no effect on $k(r)$ and k_d is not expected to influence $^{F}k_q$. This model accounts quite adequately for the lack of any pressure effects on the quenching of Rnase T_1 fluorescence by acrylamide,[41] a finding hard to explain in terms

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of unaltered migration because structural flexibility is considerably damped under these conditions.[42−44] With regard to changes in solvent viscosity, eq 7 predicts a weak dependence of ${}^{F}k_q$ in the low η_s regime ($k_d > {}^{F}k(r_p)$) and a linear dependence, ${}^{F}k_q \propto 1/\eta_s$, in the high η_s regime ($k_d \leq {}^{F}k(r_p)$). This behavior has been confirmed with RNase T_1 and parvalbumin.[4] The alternative explanation for this phenomenon, namely that on changing from low to high η_s regimes ${}^{F}k_q$ is limited initially by diffusion of the quencher through the protein and later through the solvent, does not take into account the tightening effects on protein structure of viscogenic solvents such as glycerol.[45,46] Another feature that has been considered to provide strong evidence for a penetration mechanism is the variability of ${}^{F}k_q$ among quenchers of different molecular size, such as acrylamide, succinamide, and iodide.[4] However, the nature of the quenching interaction, and consequently ${}^{F}k(r)$, differs among these molecules. Thus, I^-, which interacts through the heavy-atom effect, is expected to exhibit the shortest distance range and the smallest quenching effectiveness for buried Trp, which is in agreement with experiment. The same argument applies to the less efficient quencher succinamide. Last, much emphasis has been given to activation energies for ${}^{F}k_q$ that are generally larger than ~3 kcal mol^{-1} expected for diffusion in water.[4] Following eq 7, the influence of temperature on ${}^{F}k_q$ is to be found on the temperature dependence of ${}^{F}k(r)$ and possibly on even small variations of the distance of closest approach, r_p, neither of which is known. Perhaps of even greater concern, given the propensity of acrylamide to bind to proteins with submolar-to-molar affinities and the large concentrations needed to quench internal Trp residues, is the effect of temperature on acrylamide partitioning. In this respect, it is significant that polyhydric compounds, under similar experimental conditions, associate with protein with increased affinity at higher temperature.[47]

For Trp residues that are beyond the critical interaction distance, no fluorescence quenching is predicted to occur by eq 7. W314 of LADH is removed from the solvent by a protein layer of 4.5 Å in thickness, and the estimated ${}^{F}k_q = 10^7$ M^{-1} s^{-1} may represent a limiting case of through-space quenching. With deeper chromophores, the unusually large acrylamide concentrations needed are bound to result in considerable binding and/or perturbations of the native fold.

The vastly different acrylamide concentration range employed between fluorescence and phosphorescence quenching studies can be another factor bearing on the discrepancy between ${}^{F}k_q$ and ${}^{P}k_q$, as the artifacts associated with quencher partitioning would artificially raise ${}^{F}k_q$. However, from the above discussion it is plausible that the discrepancy originates mostly from the difference between ${}^{F}k(r)$ and ${}^{P}k(r)$. Whereas fluorescence quenching can be satisfactorily accounted for by through-space interactions, this mechanism, as noted in Table 2, is totally inefficient for phosphorescence quenching, except perhaps for residues that lie near the surface of the macromolecule. In the case of phosphorescence, the inequality ${}^{P}k_q \gg {}^{P}k_q(r_p)$ implies that migration of acrylamide to the chromophore site competes favorably with through-space interactions. Hence, ${}^{F}k_q$ and ${}^{P}k_q$ are expected to differ substantially on account of $k(r)$ and of the quenching mechanism.

In summary, the results of this study emphasize that through-space interactions are generally insignificant for quenching the phosphorescence of buried Trp residues in proteins. This feature of phosphorescence, when combined with its long, millisecond-to-second lifetime, makes it possible to monitor the slow diffusion of acrylamide to even the deepest cores of globular protein and to investigate on their dynamical structure.

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