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FLUORESCENCE QUENCHING OF THE BURIED TRYPTOPHAN RESIDUE OF COD PARVALBUMIN

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Cod parvalbumin (isotype III) is a single tryptophan-containing protein. The fluorescence characteristics of this tryptophan residue ($\lambda_{em} \approx 315$ nm) suggest that it is buried from solvent and that it is located in an apolar core of the protein. Solute quenching studies of the tryptophan fluorescence of parvalbumin reveal dynamic quenching rate constants, k_q, of 1.1×10^8 and 2.3×10^9 M^{-1} s^{-1} (at 25°C) with acrylamide and oxygen, respectively, as quenchers. From temperature dependence studies, activation energies of 6.5 ± 1.5 and 6.0 ± 0.5 kcal/mol are found for acrylamide and oxygen quenching. The k_q for acrylamide quenching is found to be relatively unchanged ($\pm 10\%$) by an 8-fold increase in the bulk viscosity (glycerol/water mixture). These temperature and viscosity studies argue that the acrylamide quenching process involves a dynamic penetration of the quencher, facilitated by fluctuations in the protein's structure.

1. Introduction

Solute quenching studies of the intrinsic tryptophanyl fluorescence of proteins have provided interesting insights regarding the dynamics of globular proteins. This is particularly true for proteins with internal tryptophan residues, for which the solute quenching process apparently involves penetration of the quencher into the protein matrix [1–4]. Molecular oxygen is a quencher which is found to be able to quench the fluorescence and phosphorescence of tryptophan residues in globular proteins with a bimolecular rate constant of $1–8 \times 10^9$ M^{-1} s^{-1} [2,4–7]. The larger and more polar quencher, acrylamide, also has the ability to quench many internal tryptophan residues (range of rate constants from $\sim 1 \times 10^8$ to 4×10^9 M^{-1} s^{-1}) [8]. However, there appear to be certain tryptophan residues which are completely (within detection limits) inaccessible to acrylamide, such as the single tryptophan resides in azurin from *Pseudomonas fluorescens* [8], asparaginase from *E. coli.* (Eftink, unpublished results), and Trp-314 in horse liver alcohol dehydrogenase [10].

For those tryptophan residues that are buried, but not completely inaccessible to acrylamide, such as the single tryptophan residue of ribonuclease T_1 from *Asperigillus oryzae*, the temperature and viscosity dependence of the solute quenching process have been found not to reflect the diffusion of acrylamide through the bulk solvent. Instead, the moderately large activation energy and the relative insensitivity to bulk viscosity characterizes the quenching process as being limited by diffusion of acrylamide through the protein matrix [3,9].

In this article we report studies of the fluorescence of cod (*Gadus callarius* L.) parvalbumin isotype III, a low molecular weight ($M_r \approx 12\,000$) Ca^{2+}-binding globular protein which has a single internal tryptophan residue (along with 10 phenylalanines and only one tyrosine) [11]. We present studies of the acrylamide and oxygen quenching of

the fluorescence of this protein, including temperature and viscosity dependence studies. The various quenching studies are performed with fluorescence lifetime measurements. Most of our previous temperature and viscosity dependence studies were based on fluorescence intensity measurements, and, thus, a purpose of the present work is to corroborate our earlier work with fluorescence lifetime measurements.

2. Experimental section

2.1. Materials

Cod parvalbumin was isolated as described by Horrocks and Collier [12] except that the final DEAE-chromatography step was eliminated. This protein was found to migrate as a single band in a denaturing polyacrylamide gel electrophoresis experiment and was found to have a characteristic, blue-shifted fluorescence emission spectrum with a maximum at 315 nm (see fig. 1).

Acrylamide was recrystallized from ethyl acetate. Glycerol and *p*-dioxane were spectral grade from Aldrich Chemical Co. On occasion glycerol was vacuum distilled before use. *N*-Acetyl-L-tryptophanamide was obtained from Sigma Chemical Co. 3-Methylindole was sublimed before use.

2.2. Methods

Fluorescence intensity and lifetime measurements were made with an SLM 4800 phase-modulation fluorometer. *p*-Terphenyl was used as a lifetime reference, as described by Lakowicz et al. [13], except for the oxygen quenching studies, for which a glycogen scattering solution was used as a reference. An excitation wavelength of 295 nm (0.5 nm slit) was used in all studies, unless specified otherwise. Emission was usually observed at 320 nm (8 nm slit) through a monochromator. For the oxygen quenching studies, emission was observed through a Corning 7-60 filter. All fluorescence intensity measurements were made relative to a reference beam in order to avoid errors due to instability or drift of the exciting beam. The fluorescence lifetimes reported here were calculated from the phase angle lag [14] using a modulation frequency of 18 MHz, unless stated otherwise.

Acrylamide and iodide quenching studies were performed by adding aliquots of a stock quencher solution to a cell containing the protein. Corrections for dilution and absorptive screening by acrylamide were made as described previously [8]. For the studies in the glycerol/water mixture, the Job method was used to mix a protein solution containing no quencher with a protein solution containing 2 M acrylamide.

Oxygen quenching studies were performed as described elsewhere [7] using a high-pressure cell borrowed from Dr. E. Gratton, University of Illinois.

Quenching data were analyzed in terms of the modified Stern-Volmer equation [1,8]

$$\frac{F_0}{Fe^{V[Q]}} = \frac{\tau_0}{\tau} = 1 + K_{sv}[Q] \qquad (1)$$

where F_0 and F are the respective fluorescence intensities in the absence and presence of quencher, Q, τ_0 and τ the fluorescence lifetimes in the absence and presence of Q, V the static quenching constant, and K_{sv} the dynamic quenching constant. The quenching rate constant, k_q, is calculated as $k_q = K_{sv}/\tau_0$.

Fluorescence yield measurements were made at 20°C with a Perkin-Elmer MPF-44A spectrophotofluorometer. Spectra were not corrected for the photomultiplier wavelength response, but a quantum yield standard, 3-methylindole in *p*-dioxane, was selected which has a similar spectral location. The emission spectra of the protein and 3-methylindole were integrated manually and compared for excitation wavelengths of 290 and 280 nm (5-nm slits). The absorbance of the solutions was approx. 0.10 for these measurements. The 3-methylindole solutions in *p*-dioxane were bubbled with argon to remove oxygen. The quantum yield determinations were corrected for the difference in the refractive index of *p*-dioxane and the aqueous buffer.

3. Results

3.1. Fluorescence of cod parvalbumin

In fig. 1 are shown the excitation and emission spectra of cod parvalbumin in phosphate buffer

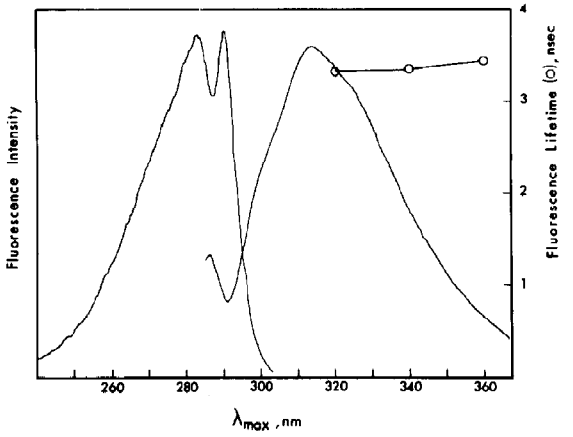

Fig. 1. Uncorrected fluorescence excitation (left) and emission (right) spectra of cod parvalbumin at room temperature, in phosphate buffer, pH 7.2. Excitation and emission slit widths, 2 nm. Also shown, the emission wavelength dependence of the phase lifetime (18 MHz).

(pH 7.2). The fluorescence λ_{max} is 315 nm. The position and shape of the emission spectrum are similar to those previously reported by Horrocks and Collier [12] for cod parvalbumin isotype III and by Permyakov et al. [16] for the analogous whiting parvalbumin isotype IIIb. The excitation spectrum shows a very prominent peak for the L_b transition at 290 nm, and has the same appearance when emission is monitored at 315 and 340 nm. The quantum yield, Φ, of cod parvalbumin is found to be 0.13 ± 0.01 at 20°C, on comparison to the standard 3-methylindole in *p*-dioxane ($\Phi = 0.66$ for standard [15]). The same quantum yield was found for the protein with excitation wavelengths of 280 and 290 nm. Also, the same quantum yield was found for the protein using *N*-acetyl-L-tryptophanamide in water as a standard ($\Phi = 0.14$ for the latter).

The fluorescence lifetime of cod parvalbumin was measured by phase-modulation fluorometry. The phase lifetime, τ_p, and modulation lifetime, τ_m, were found to be 3.37 ± 0.17 and 3.84 ± 0.16 ns, respectively, at 20°C (average of determinations for eight separate samples; modulation frequency of 18 MHz, 295 nm excitation, 320 nm emission). The fact that $\tau_m > \tau_p$ indicates that the fluorescence decay departs somewhat from a single exponential process [17]. In table 1 are shown determinations of τ_p and τ_m at 6, 18, and 30 MHz for a sample at 25°C. The variation in the values with modulation frequency also indicates that the decay is not homogeneous. We have analyzed the multifrequency data in terms of a two-component decay using the algorithm of Weber [21]. This analysis yields $\tau_1 = 1.71$ ns, $f_1 = 0.396$, $\tau_2 = 4.20$ ns and $f_2 = 0.604$.

The τ_p value (18 MHz) was found to increase slightly, from 3.3 to 3.5 ns, with an increase in the emission wavelength from 320 to 360 nm (see fig. 1). τ_p decreases with increasing temperature ($\tau_p \sim 3.5$ ns at 10°C, ~ 2.5 ns at 54°C); in fig. 2 is shown an Arrhenius plot of $\log(1/\tau_p)$ vs. $1/T$, from which an apparent activation energy of 1.5 ± 0.3 kcal/mol is obtained.

3.2. Solute quenching studies

The acrylamide quenching of parvalbumin fluorescence at 25°C is shown as the Stern-Volmer plot in fig. 3. As can be seen, there is a difference between the intensity (F_0/F) and the fluorescence lifetimes (τ_0/τ) plots indicating that both static and dynamic quenching by acrylamide occurs. From the lifetime data a dynamic quenching constant, K_{sv}, of 0.42 M^{-1} is obtained and from the relationship $K_{sv} = k_q\tau_0$, a quenching rate constant, k_q, of 1.1×10^8 M^{-1} s^{-1} is calculated. Using this K_{sv} value, eq. 1 is fitted to the intensity data to

Table 1

Frequency dependence of the apparent phase and modulation fluorescence lifetimes of cod parvalbumin

Conditions: 25°C; excitation wavelength, 295 nm: emission through a Corning 7-60 filter: standard deviations are for a set of ten 2.5-s readings on a single sample. Values in parentheses are for a binary fit to the data with $\tau_1 = 1.71$ ns, $f_1 = 0.604$, $\tau_2 = 4.20$ ns, and $f_2 = 0.296$. The latter fit is obtained by analysis of the 6 and 30 MHz data using the algorithm of Weber [21].

	Modulation frequency (MHz)		
	6	18	30
τ_P (ns)	3.23 ± 0.03 (3.20)	3.04 ± 0.01 (3.11)	3.00 ± 0.01 (2.98)
τ_M (ns)	3.76 ± 0.14 (3.64)	3.64 ± 0.01 (3.57)	3.50 ± 0.02 (3.47)

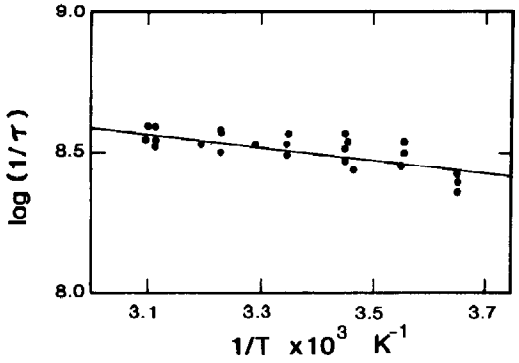

Fig. 2. Arrhenius plot for $1/\tau$ for cod parvalbumin. Fluorescence lifetimes were determined from the lag in phase angle at 18 MHz. The solid line is a least-squares fit with $E_a = 1.5 \pm 0.3$ kcal/mol.

obtain $V = 0.17\ M^{-1}$ as the static quenching constant.

There is no noticeable shift in the emission spectrum of the protein upon the addition of acrylamide. The apparent K_{sv} (from intensity measurements) at 340 nm is only about 20% larger than that at 320 nm. Part of this difference can be attributed to the slight increase in lifetime at longer wavelength. On comparison with the acrylamide quenching study of holoazurin B by Mallinson et al. [26], it is clear that our quenching data for parvalbumin cannot be accounted for in terms of the quenching of a red-fluorescing contaminant or a small amount of denatured protein.

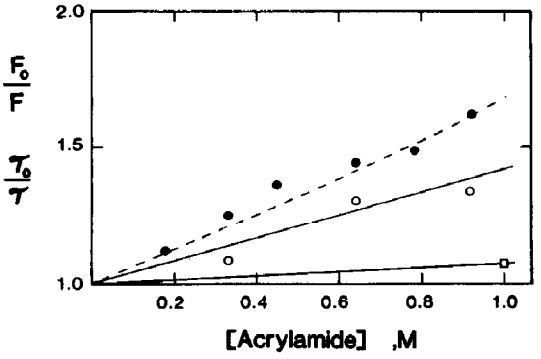

Fig. 3. Fluorescence intensity (●) and lifetime (○) Stern-Volmer plot for the quenching of parvalbumin by acrylamide at 25°C. The solid line through the lifetime data is for $K_{sv} = 0.42\ M^{-1}$. The dashed line through the intensity data is a fit with the same K_{sv} and with $V = 0.17\ M^{-1}$. Also shown, lifetime (□) data for the succinimide quenching of parvalbumin.

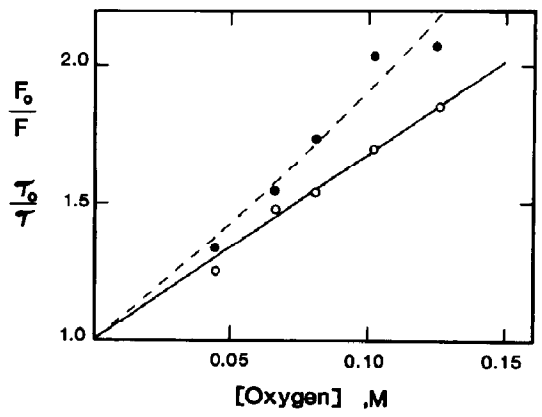

Fig. 4. Fluorescence intensity (●) and lifetime (○) Stern-Volmer plot for the O_2 quenching of parvalbumin at 25°C. The solid line through the lifetime data is for $K_{sv} = 6.8\ M^{-1}$. The dashed line drawn through the intensity data is a fit with the same K_{sv} and with $V = 1.3\ M^{-1}$.

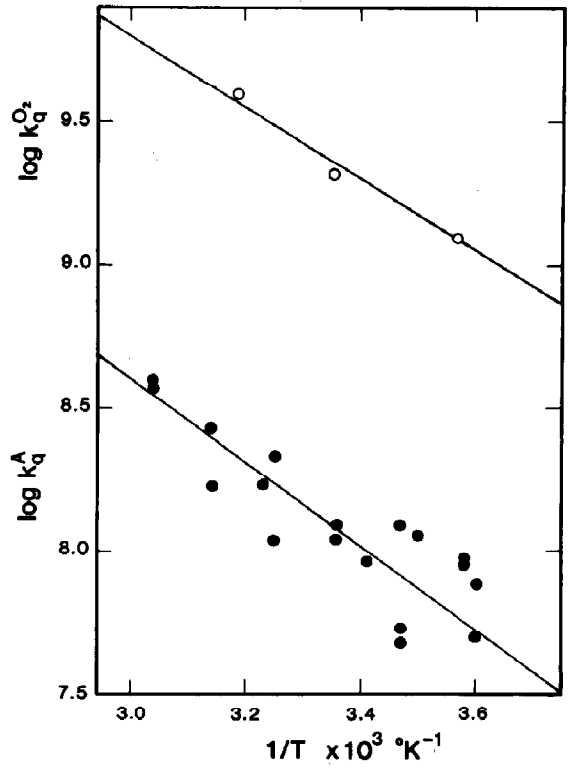

Fig. 5. Arrhenius plots for the rate constants for acrylamide (k_q^A) and oxygen ($k_q^{O_2}$) quenching of parvalbumin fluorescence. Rate constants are obtained from lifetime data. The solid lines are least-squares fits for $E_a = 6.0 \pm 1.5$ kcal/mol for acrylamide quenching and $E_a = 6.0 \pm 0.5$ kcal/mol for oxygen quenching.

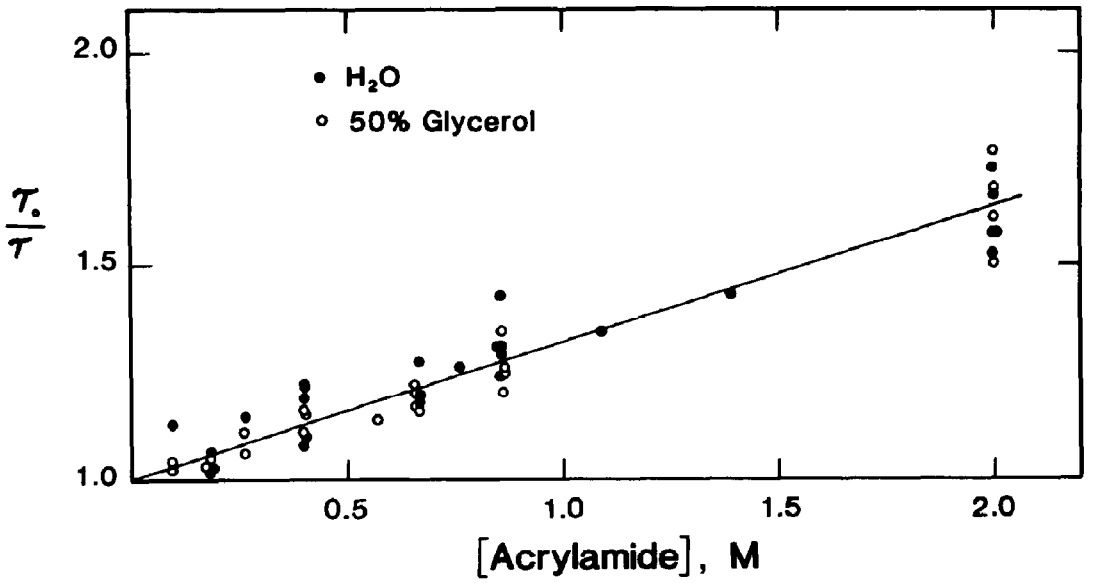

Fig. 6. Lifetime Stern-Volmer plots for acrylamide quenching of parvalbumin in an aqueous phosphate buffer (●) and a 50% (v/v) glycerol/buffer mixture (○) at 20°C. Least-squares lines having slopes of 0.33 and 0.32 are found for the aqueous and glycerol-containing solutions, respectively.

Also shown in fig. 3 are data for the succinimide quenching of parvalbumin fluorescence. For this quencher a K_{sv} of 0.07 M^{-1} is found.

In studies with KI, no significant quenching (less than 5% drop in τ_p at 0.5 M KI) was observed.

The quenching of parvalbumin fluorescence by molecular oxygen is shown in fig. 4. A dynamic quenching constant, K_{sv}, of 6.8 M^{-1} and a static quenching constant, V, of 1.3 M^{-1} are found. This dynamic quenching constant corresponds to a quenching rate constant of 2.3×10^9 M^{-1} s^{-1}.

3.3. Temperature dependence of acrylamide and oxygen quenching

The temperature dependence of the quenching of cod parvalbumin fluorescence by acrylamide and oxygen is shown in fig. 5. (The k_q values plotted were obtained from lifetime Stern-Volmer plots). Activation energies of 6.5 ± 1.5 and 6.0 ± 0.5 kcal/mol are found for acrylamide and oxygen, respectively.

3.4. Viscosity dependence of acrylamide quenching

The fluorescence quenching of cod parvalbumin by acrylamide was also studied in a 50% (v/v) glycerol/aqueous buffer mixture (~ 55% glycerol by weight; viscosity ~ 7.9 cP at 20°C). Fig 6 shows the lifetime Stern-Volmer plot of such data obtained at 20°C. Shown for comparison is the Stern-Volmer plot for acrylamide quenching in the aqueous buffer. The K_{sv} is found to be the same, within 10%, in the glycerol/water mixture and the aqueous solution. The fluorescence lifetime (τ_p) of parvalbumin in the viscous solution is 3.28 ± 0.24 ns (average of four separate determinations). which is virtually the same as that in the aqueous solution. Thus, the acrylamide quenching rate constant, k_q, is the same, within experimental error, for the glycerol/water and the aqueous protein solutions.

4. Discussion

The tryptophan-containing parvalbumin (isotype III) from codfish has not been studied by

X-ray diffraction, but the crystal structure of the homologous parvalbumin from carp has been determined [18]. The single tryptophan residue of the cod isotype III protein is believed to occur at position 102, by analogy with whiting isotype IIIb [12]. Position 102 is a phenylalanine residue in the carp protein. Assuming that the indole side chain of Trp-102 in the cod protein lies in the same position as the phenyl ring of Phe-102 in the carp protein, the indole ring of the former would lie in the central hydrocarbon-like core of the protein. This core consists of side chains of phenylalanine, leucine, isoleucine and valine residues [18].

The emission spectrum of the tryptophan residue in cod parvalbumin is relatively blue, at 315 nm, and has partially resolved vibrational structure. The emission is not quite as blue as that of the tryptophan residue in azurin [19], or as blue as that of 3-methylindole in methylcyclohexane. Still, the fluorescence λ_{max} of parvalbumin indicates that its tryptophan residue is located in an apolar microenvironment. The spectrum of parvalbumin is quite similar in position and shape to the spectrum of the single tryptophan in ribonuclease T_1 [9,20]. In addition, the activation energy for the loss of fluorescence of parvalbumin (i.e., the activation energy for $1/\tau$, which should be the same as the activation energy for $\Phi^{-1} - 1$) is one of the smallest observed for proteins [19,22] and suggests an apolar microenvironment for the tryptophan residue. The emission decay process for the protein is not homogeneous. However, the departure from a single exponential decay is not large and we have used apparent phase lifetimes to describe the fluorescence decay of the protein in most of our studies.

The solute quenching studies provide a kinetic description of the degree of exposure of this tryptophan residue in the solution state of the protein. Acrylamide and O_2 can quench the fluorescence of this residue whereas succinimide and the charged quencher, iodide, produce little or no quenching. The acrylamide and oxygen quenching rate constants (obtained from lifetime Stern-Volmer plots) are both among the smallest values that have been reported for the quenching of tryptophan residues in proteins by these respective quenchers [2,8]. The fact that the k_q for O_2 is about 20% of the diffusion-controlled limit (i.e., that for the quenching of indole in water), whereas the k_q for acrylamide is 1–2% of the diffusion-controlled limit, most likely reflects the larger size of acrylamide (see below). Also, the possible partitioning of hydrophilic O_2 molecules into the protein must be considered, in which case the k_q for O_2 will be an apparent value. A small degree of static quenching is seen for both acrylamide and O_2 quenching. Such static quenching may represent occasions in which a quencher is partitioned into the globular protein and quenches the tryptophan excited state almost instantaneously after absorption of a photon. The static quenching constants for both acrylamide and O_2 are smaller than the dynamic component and thus there is no reason to believe that there is a significant partitioning of the quenchers into the protein, at least near the site of the tryptophan residue.

The solute quenching of interior tryptophan residues is most often interpreted in terms of the dynamic penetration of the quencher into the protein matrix to strike the fluorophore [2,3]. Recently, Calhoun et al. [4], based on solute quenching studies of the phosphorescence of the interior tryptophans in horse liver alcohol dehydrogenase, have argued for a quenching mechanism involving the local unfolding of segments of the protein to expose tryptophan residues to solvent. According to this kinetic description, the apparent quenching rate constant is equal to

$$k_q(\text{app}) = \gamma k_d K_{op} \qquad (2)$$

where k_d is the diffusion-limited rate constant for encounter between quencher and a solvent-exposed fluorophore, K_{op} (an equilibrium constant describing the local unfolding process (i.e., K_{op} = [opened]/[closed]), and γ the efficiency of the quenching reaction is aqueous solution ($\gamma \approx 1.0$ for O_2, acrylamide, and iodide; $\gamma \approx 0.7$ for succinimide [23]). Thus, if acrylamide were to quench the internal tryptophan residue in parvalbumin by a segmental unfolding mechanism, the value of k_q(app) could be interpreted to yield an estimate of $K_{op} \approx 0.025$ (assuming a value of $k_d = 4 \times 10^9$ M^{-1} s^{-1}, which is approximately the value found for the acrylamide quenching of exposed tryptophan residues in large, unfolded polypeptides [8]).

Our quenching data for parvalbumin, however, are not consistent with such a local unfolding mechanism. Instead, our data support the notion that quenching occurs by inward penetration of quencher molecules. According to eq. 2 and the local unfolding model, all quenchers of parvalbumin with similar γk_d values should yield similar k_q(app) values. This is not found to be the case, as rate constants of 2.3×10^9, 1.1×10^8, $\sim 2 \times 10^7$ and $< 2 \times 10^7$ M^{-1} s^{-1} are found for O_2, acrylamide, succinimide, and iodide quenching, respectively. It is reasonable to argue that molecular oxygen, due to its apolar nature and small size, should be able to quench by a penetration mechanism, even if the other, more polar quenchers cannot [2,4]. However, it is still not possible to rationalize the 5–10-fold disparity of rate constants between acrylamide and succinimide and iodide, by the local unfolding model. This is particularly true for succinimide, which is chemically similar to acrylamide. In previous work [23] we found that, in general, succinimide is a much poorer quencher of internal tryptophan residues in proteins than expected. We demonstrated that this is due to the fact that succinimide is an inefficient quencher and that its quenching efficiency is drastically decreased on going to a non-hydrogen bonding environment. The fact that succinimide quenches the fluorescence of the internal tryptophan of cod parvalbumin with a rate constant that is 15–20% as large as that for acrylamide strongly argues that these two quenchers act by a penetration mechanism, i.e., quenching takes place in a non-hydrogen bonding (i.e., non-aqueous) environment. If an unfolding mechanism were of primary importance and quenching were to take place in an aqueous environment, one would have expected succinimide to quench with a rate constant that is about 70% as large as that for acrylamide (70% being the ratio of quenching efficiencies for the two quenchers in water).

Our observation that acrylamide quenching of cod parvalbumin is viscosity independent between 1 and 8 cP is also inconsistent with an unfolding mechanism and favors a penetration mechanism. From eq. 2, the unfolding mechanism predicts that k_q(app) will vary inversely with bulk viscosity, assuming that k_d follows the Debye relationship for a diffusion-controlled reaction (i.e., $k_d = 8RT/3000\eta$). For this not to be the case (i.e., for k_q(app) to be independent of bulk viscosity), K_{op} would have to vary fortuitously in the opposite direction with viscosity. We have also found the acrylamide quenching of other proteins with internal tryptophan residues to be relatively independent of bulk viscosity [3], so the latter explanation seems very unlikely. For the acrylamide quenching of *N*-acetyl-L-tryptophanamide or proteins with solvent-exposed tryptophan residues, we have found k_q(app) values that drop significantly when the bulk viscosity is increased. For example, with *N*-acetyl-L-tryptophanamide we have found a 5-fold drop in the acrylamide k_q with an 8-fold increase in bulk viscosity (unpublished results).

Collectively, these viscosity dependence studies and studies with various quenchers support the dynamic penetration model and are not consistent with the segmental unfolding model for the quenching process.

The moderately large activation energy (~ 6 kcal/mol) for both acrylamide and O_2 quenching of the tryptophan in cod parvalbumin further characterizes the nature of the dynamic quenching process by suggesting that the inward penetration of the probes is facilitated by small amplitude (i.e., small displacements) fluctuations in the protein on the nanosecond time scale. Similar observations of a large activation energy and insensitivity to bulk viscosity have previously been made for the acrylamide quenching of the buried tryptophan residue in ribonuclease T_1.

Our various fluorescence and fluorescence quenching studies thus characterize the tryptophan residue in cod isotype III parvalbumin as being buried within the protein structure in a nonpolar microenvironment. Other pertinent luminescence studies include the observation by Horie and Vanderkooi [24] of room-temperature phosphorescence by the tryptophan of this protein. Room-temperature phosphorescence is usually observed only for buried tryptophan residues. Quenching resolved emission anisotropy studies show the tryptophan residue in cod parvalbumin to be depolarized with an apparent rotational correlation time characteristic of global rotation of the

protein [25]. The tryptophan residue also appears to be depolarized to a minor extent by rapid segmental motion within a cone angle of about 10°. Thus the buried tryptophan residue may not be completely immobilized within the protein. Removal of the Ca^{2+} from the protein greatly changes the fluorescence properties of the protein and increases the exposure of the tryptophan residue to the solvent [16,24,25].

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