Acrylamide and Oxygen Fluorescence Quenching Studies with Liver Alcohol Dehydrogenase Using Steady-State and Phase Fluorometry†

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ABSTRACT: The fluorescence lifetime of liver alcohol dehydrogenase (LADH) has been determined by phase fluorometry at various emission wavelengths and as a function of the concentration of the quencher acrylamide. Acrylamide selectively quenches the fluorescence of the surface tryptophanyl residue Trp-15, thus allowing the fluorescence lifetime of this residue and the buried residue Trp-314 to be evaluated. Values of τ_{15} = 6.9 ns and τ_{314} = 3.6 ns are obtained, in qualitative agreement with lifetimes of these residues determined from fluorescence decay studies [Ross, J. B. A., Schmidt, C. J., & Brand, L. (1981) *Biochemistry 20*, 4369–4377]. The quenching of the fluorescence of LADH by oxygen has also been studied. Quenching by oxygen results in a blue shift in the fluorescence of the protein and a downward-curving Stern–Volmer plot. These data, along with oxygen quenching studies in the presence of 1 M acrylamide, are consistent with a model in which oxygen quenches the fluorescence of Trp-314 and -15 with quenching constants of 3.5 and 25 M^{-1}, respectively. Thus, as in studies with other quenchers, Trp-314 is found to be less accessible to the quencher oxygen than is Trp-15. A lifetime Stern–Volmer plot has also been obtained for the oxygen quenching of LADH. Such a plot deviates somewhat from the intensity Stern–Volmer plot as predicted by simulations of the quenching of two-component systems.

The fluorescence from the indole side chain of tryptophanyl residues in proteins provides a valuable intrinsic probe which can be used to gain information concerning the structural and dynamic features of the microenvironment of these residues. Such information can be obtained from analysis of the spectral position of the emission band, fluorescence decay kinetics, steady-state anisotropy and anisotropy decay kinetics, and solute quenching data for tryptophanyl residues. Much information has been gained concerning proteins from fluorescence studies with single tryptophan containing proteins (Longworth, 1968, 1981; Brochon et al., 1974; Grinvald & Steinberg, 1976; Eftink & Ghiron, 1975, 1977; Munro et al., 1979). For proteins containing more than one tryptophanyl residue, however, the analysis of the above types of fluorescence data is complicated due to the contributions from each emitter.

Liver alcohol dehydrogenase (LADH) is a protein that contains four tryptophanyl residues. Certain structural features of this protein combine to make it amenable to fluorescence studies, however. Since LADH is comprised of two identical subunits, the protein possesses only two types of tryptophanyl residues. Also X-ray crystallographic studies (Bränden et al., 1975) and various luminescence studies (Purkey & Galley, 1970; Abdallah et al., 1978; Laws & Shore, 1978; Eftink & Selvidge, 1982) indicate that Trp-15 resides on the surface of the protein, exposed to the solvent. Trp-314, on the other hand, is found to be buried quite extensively near the intersubunit contact region of the protein. Fluorescence quenching studies with I^- as quencher show Trp-15 to be selectively quenched, with essentially no quenching of Trp-314 being observed (Abdallah et al., 1978; Laws & Shore, 1978). Similarly, selective quenching of the surface tryptophan has also been observed with acrylamide as quencher (Eftink & Selvidge, 1982). The rate constant for acrylamide quenching of Trp-314 was estimated to be $\sim 10^{-7}$ M^{-1} s^{-1}. This rate constant is one of the smallest that has been measured for the quenching of a tryptophanyl residue in a globular protein (Eftink & Ghiron, 1976).

Below we report studies in which advantage is taken of the ability to selectively quench Trp-15 with acrylamide in order to determine the fluorescence lifetime of both Trp-15 and Trp-314 by using phase fluorometry. Also we report studies of the quenching of LADH fluorescence by oxygen. Since the accessibility of Trp-314 was found to be very low to ionic and polar quenchers, we were interested in determining the accessibility of this residue to the smaller quenching probe oxygen. In their oxygen quenching studies with a variety of proteins, Lakowicz & Weber (1973b) found the rate constants for the quenching of presumably buried and exposed tryptophanyl residues to be approximately the same, being 20–50% of the rate for diffusion in water. If, in the case of LADH, Trp-314 is much less accessible to oxygen than is Trp-15, this should be revealed by a downward-curving Stern–Volmer plot.

Before discussing our experimental data, we would like to present some data simulations that illustrate the effect of fluorescence heterogeneity on the fluorescence lifetime determined by phase/modulation fluorometry and on fluorescence quenching patterns. Certain of these patterns will be observed in our studies with LADH due to the heterogeneity in the lifetime and quenching rate constant for this protein.

Effect of Heterogeneity on Fluorescence Lifetimes Determined by Phase/Modulation Fluorometry. The basic principles and instrumental details of phase/modulation fluorometry have been discussed by Spencer & Weber (1969). The lifetime determined by using this technique for a heterogeneously emitting system is sometimes assumed to be equal to the weighted average lifetime, $\langle\tau\rangle = \sum_{i=1}^{n} f_i\tau_i$, where τ_i is the lifetime of each component and f_i is the fractional contribution of each component to the steady-state fluorescence intensity (at the particular excitation and emission wavelengths that the lifetime is determined). In general, however, the experimentally determined lifetime will differ somewhat from the average value for a heterogeneous system. The apparent lifetime measured from the phase lag, τ^P, will be lower than $\langle\tau\rangle$. The discrepancy between τ^P and $\langle\tau\rangle$ will increase as the difference between the lifetimes of the individual components

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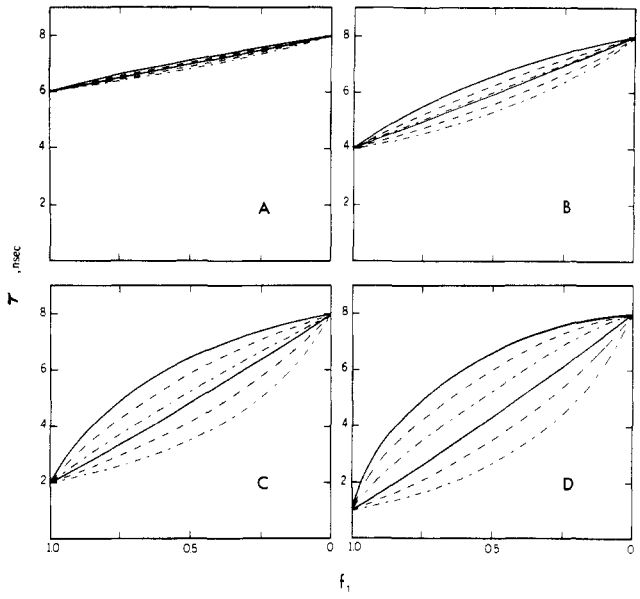

FIGURE 1: Simulated fluorescence lifetimes for two-component systems. The modulation lifetimes (upper three curves) for 6 (—), 18 (- - -), and 30 MHz (- · - ·) and the phase lifetimes (lower three curves) for 6 (—), 18 (- - -), and 30 MHz (- · - ·) were calculated from eq 1 and 2 for θ_1 and θ_2 combinations corresponding to lifetime components of 6 and 8 ns (A), 4 and 8 ns (B), 2 and 8 ns (C), and 1 and 8 ns (D).

increases and as the $\omega/\langle\tau\rangle$ ratio increases (where ω is the angular frequency of modulation of the exciting light). The apparent lifetime measured from the degree of modulation, τ^M, will usually be slightly larger than $\langle\tau\rangle$. Again the disparity between τ^M and $\langle\tau\rangle$ will be large when the difference in the lifetimes of the components is large, but the disparity decreases with increased ω. The apparent τ^P and τ^M values for a heterogeneous system are given by the following equations where θ_i represents the phase lag angles for each component and $\bar{M}$ is the average modulation defined by eq 3.

$$\tau^P = P/(Q\omega) \tag{1}$$

$$\tau^M = \left(\frac{\bar{M}^{-2} - 1}{\omega^2}\right)^{-1} \tag{2}$$

where

$$\bar{M} = (P^2 + Q^2)^{1/2} \tag{3}$$

$$P = \sum_{i=1}^{n} f_i \sin\theta_i \cos\theta_i$$

$$Q = \sum_{i=1}^{n} f_i \cos^2\theta_i$$

Spencer & Weber (1969) have previously presented data simulations demonstrating the differences between τ^P, τ^M, and $\langle\tau\rangle$. We present additional simulations in Figure 1 illustrating these differences since they bear upon the quenching studies and simulations presented below. Figure 1 shows the apparent τ^P and τ^M values that would be determined by using modulation frequencies of 6, 18, and 30 MHz for various two-component systems. From these simulations, it can be seen that τ^P measurements at 6 MHz and τ^M measurements at 30 MHz provide the truest determination of $\langle\tau\rangle$. In choosing the modulation frequency and method (phase or modulation) of lifetime measurement, one must, however, consider that random errors will lead to a greater uncertainty in lifetime measurements at 6 MHz (for lifetimes in the range of 1–10 ns; Jameson & Weber, 1981). In addition, the variation in

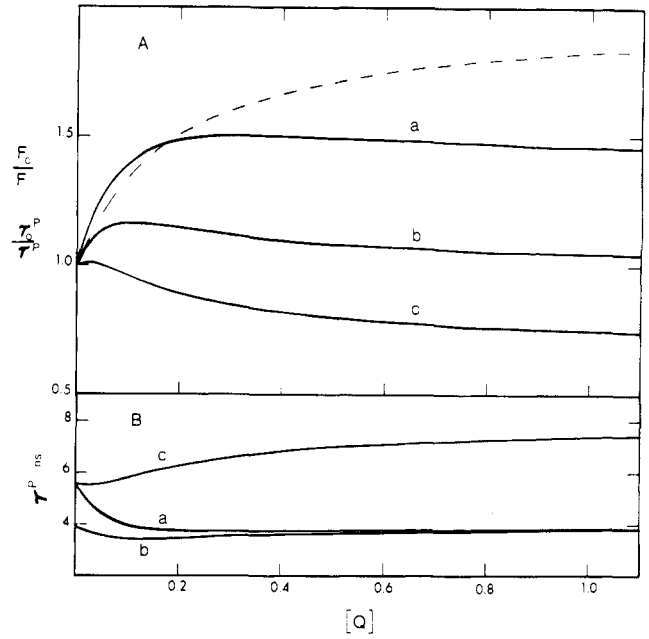

FIGURE 2: (A) Simulated Stern–Volmer plots, τ_0/τ and F_0/F, for two-component systems in which only component 1 is quenched by added quencher. The lifetimes are phase lifetime values corresponding to a modulation frequency of 18 MHz. The three solid lines are τ_0/τ plots for binary systems ($f_1 = f_2$) for which the component lifetimes are (a) $\tau_{0,1} = 8$ ns ($\theta_1 = 42.14°$) and $\tau_{0,2} = 4$ ns ($\theta_2 = 24.34°$), (b) $\tau_{0,1} = \tau_{0,2} = 4$ ns, and (c) $\tau_{0,1} = 4$ ns and $\tau_{0,2} = 8$ ns. Note that the F_0/F plot (dashed line) is the same for all three cases. (B) Plots of the phase lifetime vs. quencher concentration for the above simulated systems.

the lifetimes measured at different modulation frequencies can be used to obtain information concerning the individual component lifetimes, as discussed by Weber (1981).

Effect of Heterogeneity on the Stern–Volmer Plots Obtained from Lifetime Measurements. In fluorescence quenching studies, a coincident drop in fluorescence and lifetime is held as a criterion for the existence of dynamic quenching (Teale & Badley, 1970; Lakowicz & Weber, 1973a; Lehrer & Leavis, 1978). In addition, any difference between F_0/F and τ_0/τ plots (i.e., if the τ_0/τ values are lower than the F_0/F values) is usually taken as an indication of the extent to which static quenching takes place. Such correlations and relationships are straightforward only for homogeneously emitting systems, however. As will be demonstrated by the simulations below for heterogeneous systems (having components differing in quenching constants, K_i, and/or lifetimes, τ_i), F_0/F and τ_0/τ plots can differ considerably, even in cases where static quenching is insignificant. Lakowicz & Weber (1973b) have previously made the point that exact agreement is not to be expected for systems having a heterogeneous population of fluorophores. The simulations presented below illustrate this point for two-component systems in which one fluorophore is quenched to a much greater extent than the second. Furthermore, the lifetime values presented in the following simulations are phase lifetimes that would be measured at a modulation frequency of 18 MHz and thus include the effects of heterogeneity discussed above.

Consider, for example, the extreme cases shown in Figure 2 where the quenching constant for one component is zero ($K_1 = 0$ M^{-1}) and the other is nonzero ($K_2 = 10$ M^{-1}). F_0/F and τ_0^P/τ^P plots are compared for cases in which the unquenched lifetimes of the two components are 4 and 8, 4 and 4, and 8 and 4 ns. As can be seen, the τ_0^P/τ^P plots differ dramatically from the F_0/F plots (which are the same in each case). On replotting the simulated data as τ^P vs. [Q], one can clearly

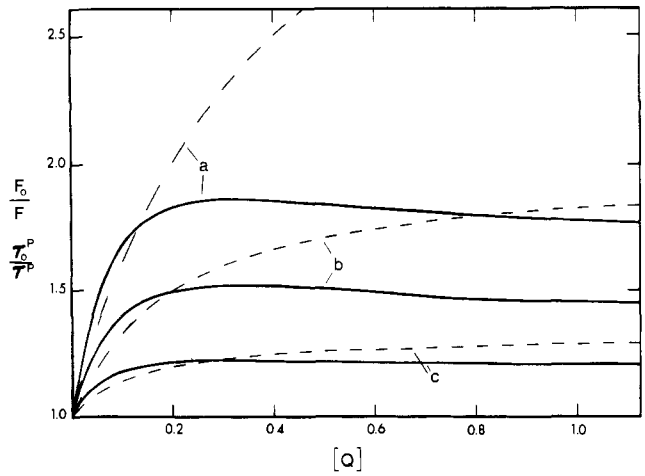

FIGURE 3: Simulated Stern–Volmer plots, F_0/F (- - -) and τ_0/τ (—), for two-component systems in which only component 1 is quenched by added quencher. The three pairs of plots correspond to cases in which (a) $f_1 = 0.75$, (b) $f_1 = 0.50$, and (c) $f_1 = 0.25$. In each case, the lifetimes of the components are $\tau_{0,1} = 8$ ns and $\tau_{0,2} = 4$ ns. The lifetimes are phase lifetimes corresponding to a modulation frequency of 18 MHz.

see that the lifetime approaches that for component 1 at high [Q].

Similar data simulations are shown in Figure 3 for the case in which f_1 is varied from 0.25 to 0.75 (for $K_1 = 0$, $K_2 = 10$ M^{-1}, $\tau_1 = 4$ ns, and $\tau_2 = 8$ ns). Again a clear difference between the F_0/F and τ_0^P/τ^P plots is seen, with the latter plots going through the maximum. For actual two-component systems such as the ones simulated here for which one component is unquenchable, the modified equation of Lehrer can be used to determine f_1, f_2, and K_2 (Lehrer, 1971; Lehrer & Leavis, 1978). Note that Lehrer's treatment is only valid for fluorescence intensity or yield measurements (i.e., plots of $F_0/\Delta F$ vs. $[Q]^{-1}$). An analogous treatment using lifetimes would not yield the true f_1, f_2, and K_2 values, and in some cases, a plot of $\tau_0^P/\Delta\tau^P$ vs. $[Q]^{-1}$ would be nonlinear.

Experimental Procedures

Materials. Both lyophilized LADH (Sigma Chemical Co.) and crystallized LADH (Boehringer-Mannheim) preparations were used. The lyophilized protein was heat treated, as described elsewhere (Eftink & Selvidge, 1982). Following this treatment, the protein sample was found to be greater than 90% active, according to the kinetic assay procedure of Dalziel (1957), and to show less tendency to aggregate than the commercial protein. The crystallized protein was dialyzed against 0.1 M phosphate, pH 7.4, buffer at 4 °C before use. Greater than 90% activity was also determined for this protein preparation by using the kinetic assay of Dalziel.

NAD^+ (grade III) was obtained from Sigma Chemical Co. Gold label trifluoroethanol (TFE) was obtained from Aldrich Chemical Co. Acrylamide was recrystallized from ethyl acetate. On some occasions, electrophoresis grade acrylamide as used without further purification.

Fluorescence Spectroscopy. Fluorescence lifetimes were measured on the cross-correlation phase fluorometer of Spencer & Weber (1969), modified with updated electronics by SLM Instruments, Inc. An emission monochromater was used in some studies; in other studies, the emission was observed through Corning 0-54 and 7-39 cutoff filters. An excitation wavelength of 295 nm (8-nm bandwidth) was used in all cases to ensure selective excitation of the Trp residues. The optical density of the solutions was less than 0.05 at this wavelength. A light modulation frequency of 18 MHz was employed.

Steady-state fluorescence measurements were made with the spectrofluorometer originally described by Weber & Young (1964), equipped with a bipolar averaging circuit (Wehrly et al., 1976). Oxygen quenching studies were performed on this instrument and the above phase fluorometer by using a stainless-steel high-pressure cell (Lakowicz & Weber, 1973a) with oxygen pressures up to 1500 psi. A 2 cm × 2 cm quartz cuvette was used in such experiments, and solutions were equilibrated (with mild stirring) for 1–1.5 h at each oxygen pressure before measurements were made. For both steady-state and lifetime oxygen quenching studies, only two or three different data points (i.e., different oxygen concentrations) were taken with a given protein solution. In this manner, fresh protein solutions were continually used. This was done to avoid long-term drift in the instruments (by making frequent F_0 and τ_0 measurements) and to avoid any complications due to slow aggregation of the protein. An excitation wavelength of 295 nm (2-nm bandwidth) was employed for these steady-state fluorescence measurements.

Steady-state acrylamide quenching studies, performed to obtain the f_i values in Table I, were done with a Perkin-Elmer MPF 44 spectrophotofluorometer, as described elsewhere (Eftink & Selvidge, 1982). All fluorescence quenching studies were performed at 20 °C.

Data Analysis. Fluorescence quenching studies were analyzed according to the Stern–Volmer relationship (eq 4) by plotting F_0/F vs. [Q], where F_0 and F are the unquenched and

$$\frac{F_0}{F} = (1 + K[Q])e^{V[Q]} \tag{4}$$

quenched steady-state fluorescence intensities and [Q] is the molar concentration of quencher. In the above equation K is the dynamic quenching constant (equal to $k_q\tau_0$, where k_q is the rate constant for quenching and τ_0 is the fluorescence lifetime of the emitter in the absence of quencher) and V is the static quenching constant. The dynamic quenching process can also be measured from the ratio of the fluorescence lifetime in the absence (τ_0) and presence (τ) of quencher as given by eq 5.

$$\frac{\tau_0}{\tau} = 1 + K[Q] \tag{5}$$

For heterogeneous systems, fluorescence quenching is described by the following equation, where f_i, K_i, and V_i are the fractional fluorescence intensity and dynamic and static quenching constants of component i.

$$\frac{F}{F_0} = \sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q])e^{V_i[Q]}} \tag{6}$$

Results

Wavelength Dependence of the Lifetime of LADH Fluorescence. The phase lifetime, τ^P, of LADH was measured at 20 °C, pH 7.0, with an excitation wavelength of 295 nm and a light modulation frequency of 18 MHz. The emission wavelength dependence of the τ^P values is given in Table I. The trend toward increasing τ^P at longer wavelengths is consistent with the data obtained by Lakowicz & Cherek (1980) also using phase fluorometry (when one considers the slightly different experimental conditions). The observed wavelength dependence of τ^P is also in agreement with the data obtained by Ross et al. (1981) using pulse fluorometry. As demonstrated by Ross et al., the increase in the average fluorescence lifetime with emission wavelength is due primarily to the heterogeneity of the fluorescence of this protein, with the

Table I: Wavelength Dependence of the Phase Lifetime of LADH and the Fractional Intensity of Trp-15

wavelength (nm)	τ^P [a] (ns)	τ^P(acrylamide) [b] (ns)	f_{15} [c]
320	4.55 (4.56)	3.57 (3.46)	0.37
330	4.60 (4.76)		0.44
340	4.99 (4.93)	3.41 (3.40)	0.49
350	5.02 (5.12)		0.54
360	5.14 (5.20)	3.40 (3.34)	0.57
370	5.28 (5.30)		0.60

[a] Values determined at 20 °C, pH 7, by using a light modulation frequency of 18 MHz. Standard deviation of the experimental lifetime values is approximately ±0.1 ns. Values in parentheses are calculated from eq 1 by using the independently determined f_{15} values in the right-hand column and θ_{15} = 38° (τ_{15}^P = 6.9 ns) and θ_{314} = 22° (τ_{314}^P = 3.6 ns). [b] Values determined in the presence of 0.91 M acrylamide; standard deviation, ±0.2 ns. The values in parentheses are calculated values for this acrylamide concentration by assuming that only Trp-15 is quenched by acrylamide and that in the absence of acrylamide θ_{15} and θ_{314} are equal to 38° and 22°, respectively. [c] Determined as described previously (Eftink & Selvidge, 1982) at 20 °C, pH 7.0, with an excitation wavelength of 295 nm and 8-nm slit bandwidths. Note that with excitation at 295 nm the f_{15} values increase with increasing excitation bandwidth. This is probably due to the fact that the absorption spectrum of Trp-15 is blue shifted with respect to Trp-314, and when the slit widths are increased, there is a greater degree of excitation into Trp-15.

red-fluorescing Trp-15 having the longer lifetime.

An estimate of the magnitude of the lifetime of each residue can be gained from the data in Table I by analysis with eq 1. The f_i values for each residue as a function of emission wavelength can be determined independently from steady-state acrylamide fluorescence quenching experiments, as demonstrated in our previous work (Eftink & Selvidge, 1982). Values for f_i are also listed in Table I. Using these values, we fitted eq 1 to the lifetime data (see Table I) for values of τ_{314}^P = 3.6 ns and τ_{15}^P = 6.9 ns. These values are in excellent agreement with the values of τ_{314} = 4.0 ns and τ_{15} = 7.4 ns obtained by Ross et al. from fluorescence decay experiments (interpolating from the data of these workers at other temperatures). This agreement is highlighted by the fact that different lifetime instrumentation (phase fluorometry and pulse fluorometry) is used in the two studies.

Note that this analysis assumes that τ_{314} and τ_{15} do not vary with emission wavelength. In studies with the single tryptophan containing proteins melittin and human serum albumin, Lakowicz & Cherek (1980) have found an increase in the phase lifetime of the tryptophan fluorescence of these proteins with increasing emission wavelength. These workers attributed this trend as being due to the dipolar relaxation processes taking place within the microenvironments of the tryptophanyl residues on the nanosecond time scale. However, in studies with ribonuclease T_1, a protein containing a single, highly buried, blue-emitting tryptophanyl residue, we have found its fluorescence lifetime to be independent of emission wavelength (τ^P = 3.73 ± 0.09 and 3.68 ± 0.15 ns at 325 and 360 nm, respectively) (M. R. Eftink and D. M. Jameson, unpublished results). Taking the tryptophanyl residue of ribonuclease T_1 as a model for the buried residue, Trp-314, in LADH, one would expect the lifetime of the latter to be independent of emission wavelength. Moreover, the fluorescence decay analyses of LADH by Ross et al. (1981) are consistent with the lifetime of Trp-314 being independent of emission wavelength and with that of Trp-15 being only slightly wavelength dependent. We believe, therefore, that our assumption of emission wavelength independence in analyzing our phase lifetime data to obtain τ_{314} and τ_{15} is valid to a first approximation. Also, as reported below, selective acrylamide quenching studies confirm our lifetime assignments for the two residues.

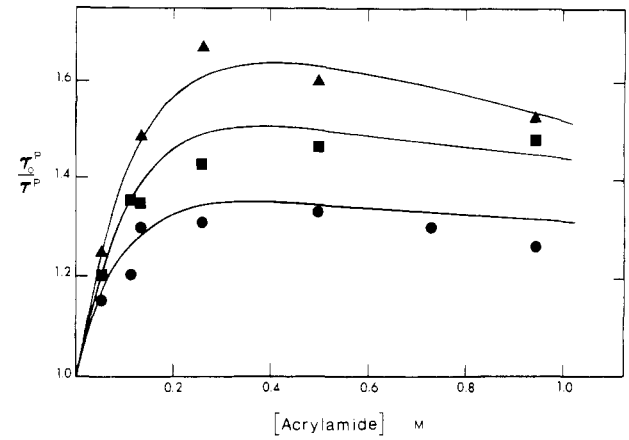

FIGURE 4: Phase lifetime Stern–Volmer plots for the acrylamide quenching of LADH (lyophilized, heat-treated sample) at 20 °C, pH 7, 0.03 M phosphate buffer, for emission wavelengths of 320 (●), 340 (■), and 360 (▲) nm. Excitation wavelength, 295 nm; excitation slits, 8 nm; emission slits, 8 nm. The solid lines are fits of eq 1 to the data by assuming $\tau_{0,314}$ = 3.6 ns (θ_{314} = 22°), $\tau_{0,15}$ = 6.9 ns (θ_{15} = 38°), and f_{314} in the absence of quencher to be 0.63, 0.52, and 0.43 at 320, 340, and 360 nm, respectively. In the presence of quencher, f_{314} and f_{15} were calculated by assuming that only Trp-15 is quenched by acrylamide with a dynamic quenching constant of 7.5 M^{-1} and a static quenching constant of 0.75 M^{-1}, using the Stern–Volmer equation (i.e., eq 6) (Eftink & Selvidge, 1982). The lifetime of Trp-15 in the presence of a given quencher concentration was also calculated by using the Stern–Volmer equation by assuming a dynamic quenching constant of 7.5 M^{-1}. The τ_{15} values were converted to θ_{15} values for use with eq 1 by interpolation from a table of θ vs. τ values at 18 MHz.

Fluorescence Lifetime Studies of the Acrylamide Quenching of LADH. The τ^P of LADH at three different emission wavelengths was determined as a function of acrylamide concentration. The data, plotted in Stern–Volmer fashion, are shown in Figure 4. As can be seen, the Stern–Volmer plots, particularly for the 320- and 360-nm data, appear to show maxima as predicted by the simulations (compare to Figure 3). The data also show a greater extent of quenching at longer emission wavelength, consistent with the red-fluorescing Trp-15 being the residue preferentially quenched by acrylamide (Eftink & Selvidge, 1982). At all three emission wavelengths studied, a lifetime of 3.4–3.6 ns was approached at high [acrylamide]. These limiting τ^P values (for 0.91 M acrylamide) are also listed in Table I as a function of wavelength. This concentration of acrylamide preferentially quenches ~ 88% of the fluorescence of Trp-15 at the three fluorescence emission wavelengths, and the limiting τ^P value of 3.4–3.6 ns can be taken as a first approximation of τ_{314}^P. Since the quenching of Trp-15 is not entirely complete, one can estimate that τ_{314}^P should be approximately 0.1–0.2 ns longer than the above value (since acrylamide is primarily a dynamic quencher, the residual fluorescence from Trp-15 will have a reduced lifetime, and its contribution will lower the apparent τ^P value in the presence of 0.91 M acrylamide). Taking this into account, we estimate that τ_{314}^P is 3.6 ± 0.2 ns. Again from the f_i values in Table I, τ_{15}^P can then be estimated to be 6.9 ± 0.4 ns. The solid lines in Figure 4 show fits to the data for these values of τ_{15} and τ_{314} and the f_i values given in Table I (see legend of Figure 4 for details). Thus, a second estimate of the lifetimes of the two types of tryptophanyl residues of LADH is obtained by taking advantage of the selective quenching of Trp-15 by acrylamide. Again the above values are in agreement with those found by Ross et al. (1981) from

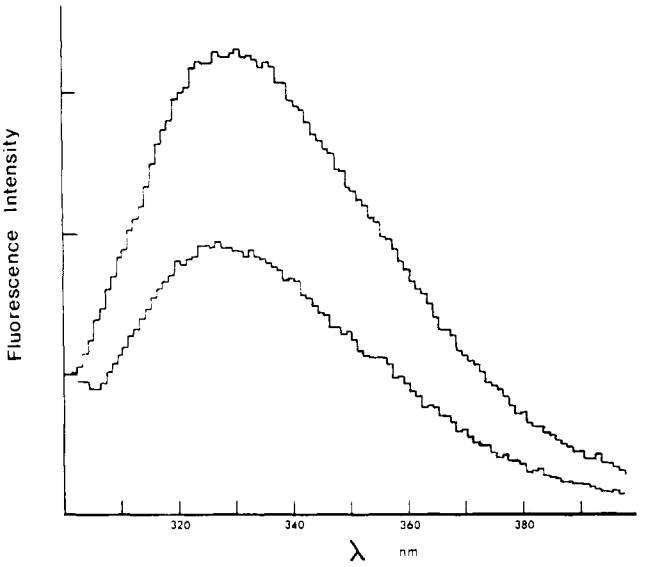

FIGURE 5: Technical emission spectra of LADH (crystallized sample) in the absence (upper spectrum) and presence (lower spectrum) of 0.116 M O_2. Excitation wavelength, 295 nm; excitation bandwidth, 2 nm; emission bandwidth, 8 nm. Conditions: pH 7, 0.03 M phosphate buffer, 20 °C.

fluorescence decay experiments. Our lifetime values disagree, however, with those assigned by Barboy & Feitelson (1978). These workers assigned the long-lifetime component to Trp-314. Our lifetime measurements in the presence of acrylamide (and the measurements of Ross et al. in the presence of KI) clearly show Trp-314 to be the short-lifetime fluorescence component.

Oxygen Quenching of LADH Steady-State Studies. The steady-state fluorescence quenching of LADH by oxygen was studied at pH 7.0, 20 °C. Figure 5 shows the emission spectrum of LADH in the absence and presence of 0.116 M O_2 (1240 psi). A blue shift is observed on quenching (from 330 to 326 nm), indicating that there is preferential quenching of the surface Trp-15. This is also demonstrated by the Stern–Volmer plots (Figure 6A) showing a larger slope at the red edge of the emission spectrum than at the blue edge. Furthermore, the Stern–Volmer plots show noticeable downward curvature indicative of selective quenching, illustrated clearly by the data for an emission wavelength of 330 nm in Figure 6B. The Stern–Volmer plots do not turnover as sharply as those for which acrylamide or iodide is used as a quencher. This indicates that the selectivity in quenching by oxygen is not as great as with these quenchers. That is, the oxygen quenching constant for Trp-314 is probably much larger than the acrylamide and iodide quenching constants for this residue.

In order to dissect the oxygen quenching constants for both residues (and to conclusively demonstrate that the oxygen quenching constant for Trp-314 is much less than that for Trp-15), we performed an oxygen quenching experiment in the presence of 1 M acrylamide (double quenching experiment). As we have shown (Eftink & Selvidge, 1982), acrylamide quenches primarily the fluorescence of Trp-15. At a concentration of 1 M acrylamide, the fluorescence of Trp-15 will be reduced to ~11% of its original value (its value in the absence of quencher). This residual Trp-15 fluorescence will be quenched by oxygen, but the effective oxygen quenching constant of this residue will be lower than the value in the absence of acrylamide by the factor $K_{15}^{O_2}/(1 + K_{15}^{A}[A])$, where $K_{15}^{O_2}$ and K_{15}^{A} are the oxygen and acrylamide quenching constants of Trp-15 (K_{15}^{A} is approximately 7.5 M^{-1} at 20 °C, and $K_{15}^{O_2}$ is approximately equal to 25 M^{-1}, vide

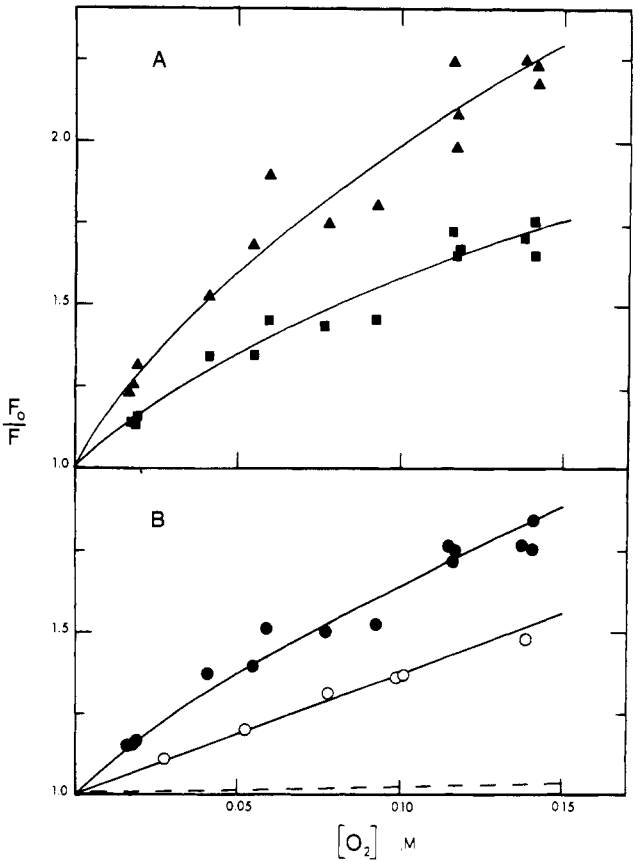

FIGURE 6: (A) Stern–Volmer plots for the O_2 quenching of LADH (crystallized sample) at emission wavelengths of 320 (■) and 370 nm (▲). Conditions same as in Figure 5. (B) Same as above for the O_2 quenching of LADH with emission at 330 nm in the absence (●) and presence (○) of 1 M acrylamide. The dashed line represents the expected extent of quenching by O_2 of the residual fluorescence of Trp-15 in the presence of 1 M acrylamide. The solid line through the data in the absence of acrylamide is a fit of eq 6 to the data for values of $K_{15}^{O_2}$ = 25 M^{-1}, $K_{314}^{O_2}$ = 3.5 M^{-1}, f_{15} = 0.29, and f_{314} = 0.71. The values of f_{15} and f_{314} were estimated independently from acrylamide quenching studies by using identical wavelength and bandwidth settings.

post) and [A] is the concentration of acrylamide. The dashed line in Figure 6B illustrates the expected amount of oxygen quenching due to residual Trp-15 fluorescence.

The experimentally observed quenching by oxygen in the presence of acrylamide is shown as the open symbols in Figure 6B. The amount of quenching above that expected for the quenching of the residual Trp-15 fluorescence can be assigned to the quenching of Trp-314. An oxygen quenching constant for Trp-314 of 3.5 M^{-1} is found. When the lifetime of 3.6 ns for Trp-314 is taken into consideration [see above, and the work of Ross et al. (1981)], an effective rate constant for oxygen quenching of Trp-314 of 1.0×10^9 M^{-1} s^{-1} is calculated. This rate constant is 50% smaller than the lowest oxygen quenching rate constants reported by Lakowicz & Weber (1973b) for tryptophanyl residues in other proteins.

With knowledge of $K_{314}^{O_2}$, the value of $K_{15}^{O_2}$ can also be calculated. To do this, we make use of values of f_{15} and f_{314}, the fractional fluorescence intensities of the two residues, obtained from acrylamide quenching studies. At 20 °C and with an excitation and emission wavelength of 295 (2-nm slits) and 330 nm (8-nm slits), values of f_{15} = 0.29 and f_{314} = 0.71 are found. From these values and the above value of $K_{314}^{O_2}$, a $K_{15}^{O_2}$ value of 25 M^{-1} is determined. The solid curve through the data in Figure 6B (in absence of acrylamide) shows the fit of eq 6 to the data provided by these quenching parameters.

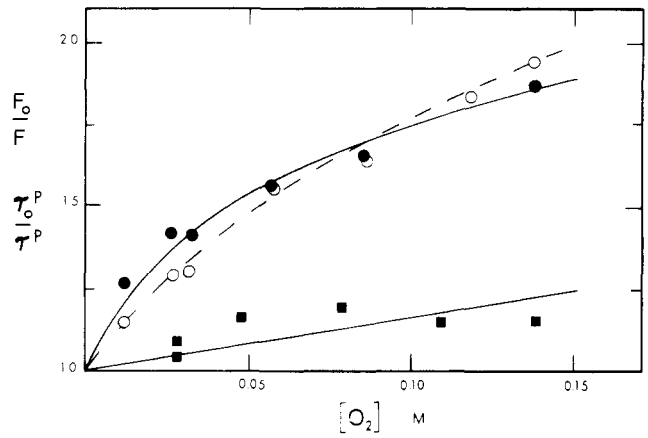

FIGURE 7: Lifetime (●) and intensity (○) Stern–Volmer plots for the O_2 quenching of LADH (lyophilized, heat-treated sample, pH 7.0 0.02 M Tris–HCl/0.08 M NaCl buffer). Stern–Volmer plot for the O_2 quenching of LADH in the presence of 0.89 M acrylamide (■). Lines do not represent theoretical fits. The dashed line emphasizes the difference in the lifetime and intensity quenching profile for LADH. Excitation wavelength, 295 nm (8-nm bandwidth); emission observed through Corning 0-54 and 7-39 filters (resulting in an observed emission band centered at 345 nm).

From this $K_{15}^{O_2}$ value and the $\tau_{0,15}$ value of 6.9 ns, the rate constant for O_2 quenching of Trp-15 is found to be 3.5×10^9 M^{-1} s^{-1}. This value is in the range of values previously reported by Lakowicz & Weber (1973b) for other proteins.

Oxygen Quenching of LADH Lifetime Studies. The oxygen quenching of LADH was also studied by phase lifetime measurements. Figure 7 shows a τ_0^P/τ^P vs. $[O_2]$ plot obtained with an excitation wavelength of 295 nm and observation of the emission through cutoff filters (Corning 0-54 and 7-39). A downward curvature is seen. Also in Figure 7 is shown a plot of the fluorescence intensity ratio vs. $[O_2]$. The fluorescence intensity values were obtained from direct-current voltage reading on the phase fluorometer on the same sample used for the lifetime measurements. The F_0/F plot does not curve as sharply as the τ_0^P/τ^P plot, consistent with the pattern predicted in Figures 2 and 3 for a case in which heterogeneous quenching occurs (i.e., compare to Figure 3, curve b). A τ_0/τ plot for the oxygen quenching of LADH in the presence of 0.89 M acrylamide is also shown in Figure 7. Although there is much scatter in the data (due to low fluorescence intensity of the sample in the presence of the high concentration of acrylamide and the small changes in the observed lifetimes), an oxygen quenching constant for Trp-314 of approximately 1.5 M^{-1} can be estimated. The fact that this quenching constant is lower than the value of $K_{314}^{O_2}$ estimated from steady-state intensity measurements (Figure 6B) suggests that a certain degree of static quenching of this residue by oxygen may occur. Additional studies will be necessary in order to clarify this matter.

Discussion

The present work is the first demonstration of selective quenching by O_2 of the intrinsic tryptophanyl fluorescence of a globular protein. In their pioneering studies with over a dozen different proteins, Lakowicz & Weber (1973b) found O_2 to quench tryptophanyl fluorescence with a rate constant ranging from 2×10^9 to 5.4×10^9 M^{-1} s^{-1}. A slight blue shift on quenching was observed by these workers for certain proteins, but all Stern–Volmer plots were reported to be linear.

For LADH, we observe a significant blue shift on O_2 quenching and a clear downward curvature in the Stern–Volmer plot. It should be pointed out that in general it will be difficult to see downward-curving Stern–Volmer plots in quenching studies with O_2 due to the limited concentration range (0–0.15 M) normally employed. In contrast, in studies with acrylamide or iodide, up to 1 M concentration of quencher can be used, thus making possible the achievement of more pronouncedly curved Stern–Volmer plots. The fact that a clear downward curvature can be seen in our O_2 quenching data with LADH indicates that the quenching constant for the two components must differ by a factor of more than 4. This difference in quenching constants between Trp-15 and -314 is due at least partially to the fact that the fluorescence lifetime of the surface residue, Trp-15, is almost twice as long as that of Trp-314. The rate constants for the O_2 quenching of these two residues are also different. From the O_2/acrylamide double quenching experiment, the O_2 quenching constants for Trp-15 and -314 are assigned as 25 and 3.5 M^{-1}, respectively. These constants along with the lifetimes of the two residues allow calculation of the O_2 quenching rate constants, k_q, to be 3.5×10^9 and 1.0×10^9 M^{-1} s^{-1} for Trp-15 and -314. The value for Trp-15 is typical for tryptophanyl residues in proteins, but the k_q for Trp-314 is significantly smaller than any previously determined value. Since the k_q values for acrylamide and iodide quenching of Trp-314 are extremely small ($\sim 10^7$ M^{-1} s^{-1} for acrylamide and perhaps smaller for iodide), the low quenching rate constant for O_2 is not unexpected.

On the other hand, one can take the point of view that the rate constant for O_2 quenching of Trp-314 is still quite large for a Trp residue that has the remarkable ability to phosphoresce at room temperature (Saviotti & Galley, 1974) and that is shown by X-ray crystallography to be deeply buried at the subunit interface of this protein (Bränden et al., 1975). Our observed quenching rate constant for Trp-314 is actually only a factor of 10 smaller than the rate constant for diffusion of O_2 through water. This can be viewed as support for the proposal of Lakowicz & Weber (1973b) that O_2 is readily able to permeate the structure of globular proteins as a result of nanosecond structural fluctuations in the latter. Since most of the proteins studied by Lakowicz and Weber in their pioneering O_2 quenching experiments were multi-Trp-containing proteins (thus giving only average quenching rate constants), our study with LADH strengthens the above proposal by our assignment of an individual rate constant for the quenching of the interior Trp residue in this protein. We note also that the O_2 quenching of Trp-314 does not occur via a migration of energy (resonance energy transfer) from Trp-314 to the more exposed Trp-15. The fact that the Stern–Volmer plots for acrylamide and iodide quenching of LADH are downward curving (Eftink & Selvidge, 1982; Abdallah et al., 1978) precludes this possibility and indicates that the quenching of Trp-314 must involve either (a) the penetration of the quenching species into the protein matrix to strike this interior residue or (b) transient unfolding-type fluctuations in the protein structure leading to the exposure of Trp-314 to the solvent.

The fact that the rate constant for O_2 quenching of Trp-314 is approximately 100 times larger than that for the quenching of this residue by acrylamide (1×10^9 M^{-1} s^{-1} vs. $\sim 10^7$ M^{-1} s^{-1}) lends support to the first possibility listed above. Considering the difference in size and polarity of these two quenchers, one would expect O_2 to be able to diffuse into a protein matrix much more readily than acrylamide (Eftink & Ghiron, 1981).

It is also of interest to compare the magnitude of our rate constant for the O_2 quenching of Trp-314 fluorescence with the data of Saviotti & Galley (1974) for the O_2 quenching of

the room-temperature phosphorescence of Trp-314. Our rate constant is approximately 1500-fold larger than the O_2 phosphorescence quenching rate constant reported by these workers. The basis for this discrepancy is unclear (Lakowicz, 1980). However, some recently reported room-temperature phosphorescence studies with LADH by Vanderkooi and coworkers (Vanderkooi et al., 1982) may help resolve matters. The latter workers have reported an O_2 phosphorescence quenching rate constant of the same magnitude as our fluorescence quenching rate constant.

The present studies also demonstrate that phase/modulation fluorometry can be used to obtain the individual lifetimes of a heterogeneously emitting system. The lifetimes we have obtained for Trp-15 and -314 of LADH agree quite well with the values determined previously by Ross et al. (1981) from fluorescence decay studies. In assigning the lifetimes of the two tryptophanyl residues from our phase lifetime data, we took advantage of the ability of acrylamide to selectively quench the fluorescence of Trp-15. For most proteins, it will probably not be possible to selectively quench certain tryptophanyl residues, as can be done with LADH. However, as recently discussed by Weber (1981) and demonstrated in work by Jameson & Weber (1981), the component lifetimes of a heterogeneous system can in principle be determined from measurements of the phase modulation lifetime of such a system by using appropriate modulation frequencies.

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