Lifetimes and NADH Quenching of Tryptophan Fluorescence in Pig Heart Lactate Dehydrogenase†

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ABSTRACT: The decay of tryptophan emission from pig heart lactate dehydrogenase following pulsed excitation has been recorded in Tris buffer solution at pH 7.4. All tryptophan residues emit. A good least-squares two-component fit is obtained with $I(t) = 0.53e^{-t/1.2} + 0.47e^{-t/6.8}$. A longer lived emitter (τ = 7.4–8.1 ns) is also observed. Bound NADH strongly quenches most of the 6.8-ns emission, but the 1.2-ns component is relatively unaffected. The fluorescence is moderately quenched by acrylamide and only slightly quenched by I^- and Cs^+. The pulsed and steady-state fluorescence is discussed in terms of a model with three lifetime classes of tryptophan, viz., 1, 4, and 8 ns. The three-dimensional structure of the enzyme–NADH complex is used to develop a description of the individual residues in terms of their lifetimes and sensitivity to NADH and I^- quenching. The nonlinear NADH quenching is due to intersubunit energy transfer from Trp-248 to NADH.

Fluorescence measurements have been used widely to obtain information about protein structure and conformational changes induced by alteration of the environment and/or ligand binding. Most proteins contain more than one tryptophan, and assessment of the contribution of each tryptophan to the total emission is requisite for a complete analysis of environmental or ligand perturbation. The complexity of such an analysis is well illustrated by lysozyme, a monomeric enzyme with six tryptophans, only two of which are responsible for the bulk of the fluorescence (Formoso & Forster, 1975).

Considerable attention has been directed to the study of the fluorescence of the dehydrogenases. Of particular interest is lactate dehydrogenase, which in higher animals is a tetrameric molecule, with four identical NADH binding sites. The tryptophan fluorescence yield of the fully complexed enzyme is 14% of that of the free enzyme. Most, if not all, of the quenching is due to Trp → NADH energy transfer. The extent of quenching by NADH depends nonlinearly on the extent of complex formation. The nonlinearity arises from competition between two or more NADH acceptors for excitation energy localized on a single tryptophan donor, i.e., from intersubunit quenching, and has been termed "geometric" quenching (Stinson & Holbrook, 1973).

The quenching process in ox heart lactate dehydrogenase[1] has been modeled for several geometries by assuming that all quenchable tryptophans have identical fluorescence yields and hence lifetimes (Owens & Teale, 1976). The residual fluorescence was attributed to tryptophans that are distant from all NADH molecules. Good concordance between calculated and experimental quenching curves was considered to support the assumptions of the model. Since the decay constants of the several tryptophans are likely to differ, pulsed excitation can be useful in sorting out the contributions of the individual tryptophans to the overall emission. Our results with pig heart LDH show that there are at least three classes of tryptophans, two being quenchable by NADH, and that the residual fluorescence includes a contribution from some of the quenched residues.

The three-dimensional structure of dogfish LDH has been published (Holbrook et al., 1975), the sequence of pig heart LDH has been determined (Eventoff et al., 1977), and the NADH–Trp separations in dogfish LDH have been calculated (Holbrook et al., 1975). The disposition of the six invariant tryptophans relative to NADH is apparently the same in the two species. Because of this and the substantial literature on pig heart LDH, this enzyme is an attractive subject for learning how to utilize fluorescence measurements to sort out the individual chromophore contributions in proteins with three or more emitters. A principal conclusion from the results to be described is that time-resolved fluorescence measurements can be used, in conjunction with structural information, to give a partial interpretation of the fluorescence from a protein as large as LDH with six emitters. It is noteworthy that several features of this interpretation are independent of the structural information.

Experimental Procedures

Materials. The H_4 isozyme of pig heart LDH was purified by a modification (M. Rossmann, private communication) of a published procedure (Reeves & Fimognari, 1966). After initial extraction and fractional precipitation, the enzyme was passed through a DEAE–cellulose column (Whatman DE-52) at 4 °C. The eluant was precipitated with ammonium sulfate, centrifuged, redissolved in 0.10 M sodium phosphate buffer of pH 7.0, and applied to an affinity column (5′-AMP–Sepharose 4B, Pharmacia) that had been equilibrated with the phosphate buffer. Elution was with a linear NADH gradient (0–(1.1 ± 0.1) × 10^{-4} M NADH in the phosphate buffer). The purified enzyme was stored as a precipitate in 70% saturated $(NH_4)_2SO_4$. Before use, the enzyme was treated with charcoal (Heck, 1969) and dialyzed overnight.

Polyacrylamide gel electrophoresis (Dietz & Lubrano, 1967; Fritz et al., 1970) showed that other isozyme concentrations were about 0.5% of the H_4-enzyme level. The purified material had A_{280}/A_{260} = 1.98 and a specific activity of 470 units/mg, measured in 67 mM sodium phosphate buffer solution of pH 7.2 (a unit of activity corresponds to oxidation of 1 mol of NADH/min at 30 °C in a mixture containing 0.36 mM pyruvate and 1.55 × 10^{-4} M NADH).

NADH (P-L Biochemicals, Inc.) was purified immediately prior to use by DEAE-cellulose column chromatography using a linear 0.1–0.3 M NH_4HCO_3 gradient (Mueggler et al., 1975). The purified material had A_{260}/A_{340} = 2.26.

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[1] Abbreviation used: LDH, lactate dehydrogenase.

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Samples for fluorescence measurements were dissolved in 0.02 M Tris buffer of pH 7.4, and the total ionic strength was adjusted to 0.3 with NaCl. All LDH concentrations refer to active-site concentrations. These ranged from 10^{-6} to 10^{-7} M and were calculated using a molar extinction coefficient of 194 000 M^{-1} cm^{-1} for the tetramer at 280 nm.

Steady-State Fluorescence Measurements. Fluorescence intensities were measured with an Aminco-Bowman spectrofluorometer thermostatted at 26 ± 0.1 °C and fluorescence spectra with an instrument assembled in this laboratory and used at room temperature (24 ± 1 °C). Steady-state intensities (F) were used to determine two quantities: (1) the NADH binding constants and (2) the extent of tryptophan quenching for LDH with bound NADH.

Binding Constants. For this determination only NADH, bound and free, was excited by 340-nm radiation. The total NADH emission was monitored at 450 nm. The difference in fluorescence intensity (ΔF) between a solution of LDH and NADH and one containing only NADH at the same total NADH concentration is a measure of the bound NADH, providing several conditions are fulfilled. These are (1) the NADH binding sites are optically independent, i.e., the fluorescence quantum yield of each complexed NADH is the same; (2) the molar extinction coefficients of free and complexed NADH are equal; and (3) the ΔF values have been corrected for inner filter effects. The independence of the four active sites and evidence for the applicability of a single dissociation constant have been presented by Stinson & Holbrook (1973). The quantum yield ratio, Φ_{bound}/Φ_{free}, exceeds 5 for NADH, and only a large variation in molar extinction coefficients (>10%) would vitiate the second assumption. The correction factor for inner filter effects has been given by Parker (1968) as

$$\frac{F_{cor}}{F} = \frac{2.303A(d_2 - d_1)}{10^{-Ad_1} - 10^{-Ad_2}}$$

where A is the total absorbance at the exciting wavelength and d_1 and d_2 define the geometry of the "observed" volume. For the NADH concentrations used in this work ($A < 0.2$) multiplication of ΔF by $10^{A/2}$ is sufficiently accurate to make ΔF_{cor} proportional to [EL]. When this correction was applied, the fluorescence intensity of NADH was a linear function of NADH concentration to $A = 0.6$.

The dissociation constant can be expressed in terms of the total ligand and binding site concentrations, $[L]_0$ and $[E]_0$, and the fractional occupation of the binding sites α.

$$\alpha = \frac{[EL]}{[E]_0} = \frac{\Delta F}{\Delta F_{max}}$$

These quantities are related to the dissociation constant by the equation

$$K_d = (1 - \alpha)\left(\frac{[L]_0}{\alpha} - [E]_0\right) \quad (1)$$

ΔF_{max} is proportional to the concentration of fully bound complex ($[E]_0$). K_d is very sensitive to the choice of ΔF_{max}, which cannot be estimated independently with sufficient accuracy because the correction described above is not reliable at high NADH concentration. Consequently, ΔF_{max} was treated as a free parameter in a least-squares fitting program that is used to evaluate K_d and ΔF_{max} from eq 1 and the fluorescence data.

Quenching of LDH Emission by NADH. Tryptophan was excited at 295 nm and the emission detected at 340 nm. The emission spectrum (λ_{max} 337 nm) does not change shape or position as NADH is added. Therefore intensity changes at a single wavelength reflect the fluorescence quantum yield variation. In addition to the inner filter effect described above, fluorescence reabsorption can be important. When NADH emission is monitored at 450 nm, the reabsorption is negligible, but the LDH emission spectrum overlaps the NADH absorption spectrum, and reabsorption of tryptophan fluorescence cannot be ignored. Rather than attempting to calculate the reabsorption by assuming an uncertain geometry, we have used an empirical procedure wherein the fluorescence of a standard (*N*-acetyltryptophanamide) is measured as a function of added NADH. The optical densities of LDH and *N*-acetyltryptophanamide were the same. The lifetime of *N*-acetyltryptophanamide is not affected by NADH up to 10^{-4} M, and consequently the intrinsic emission of *N*-acetyltryptophanamide is not quenched by NADH. Any apparent reduction in emission intensity is then due to inner filter and reabsorption effects, and such reductions were used to correct enzyme fluorescence data. The emission spectra of *N*-acetyltryptophanamide and LDH are slightly different, but the error in the correction due to this wavelength difference is small.

Lifetime Measurements. The emission was excited by pulses (295 nm) from an ADP-doubled Rhodamine 6G dye laser pumped by an Avco C-950 N_2 laser (Andrews et al., 1974), and the intensity profiles were recorded by a current sampling method. The lifetimes were obtained by convoluting a two-component decay function, $I(t) = a_1e^{-t/\tau_1} + a_2e^{-t/\tau_2}$, with the excitation pulse, using least-squares fitting routines with either truncated Taylor's series or Simplex search procedures. Precision in τ_1 and τ_2 was usually ±0.1 ns, while the a_2/a_1 reproducibility was generally better than 10% for a particular sample. Scattered light was negligible and the decay was not dependent on the polarization plane of the exciting light. The measurements were made at room temperature (24 ± 1 °C).

Results

NADH Binding Constants. In order to assess quantitatively the effect of NADH binding on LDH fluorescence, reliable binding constants must be available. Since temperature and buffer composition markedly affect NADH binding (Stinson & Holbrook, 1973), we determined the binding constants under the conditions used for the fluorescence quenching measurements in this work, viz., pH 7.4, Tris buffer of 0.3 ionic strength, and 26 °C. The K_d values ranged from 3×10^{-6} to 5×10^{-6} M. The best value, $(4.2 \pm 0.2) \times 10^{-6}$ M, is higher than the one reported by Stinson & Holbrook (1973), 0.5×10^{-6} M (pH 7.2, 67 mM sodium phosphate buffer). However, it is nearly the same as that found at pH 7 in 0.2 M potassium phosphate buffer solution (Schmid et al., 1976).

LDH Fluorescence Lifetimes. Typical excitation and emission intensity profiles are shown in Figure 1. The convolution obtained from the two-component least-squares fit, $I(t) = 0.53e^{-t/0.94} + 0.47e^{-t/6.79}$, is also shown. Lifetime data were collected over a period of many months, and while the reproducibility in τ_1, τ_2, and a_1/a_2 was generally good on any given day, some temporal variation was encountered. The limits of this variation are represented by $I(t) = 0.63e^{-t/0.62} + 0.37e^{-t/6.81}$ and $I(t) = 0.52e^{-t/1.41} + 0.48e^{-t/6.49}$. This variation, which may be due to changes in the protein or artifacts in the data collection system, does not affect the conclusions to be presented.

Quenching of LDH Fluorescence by NADH. The steady-state fluorescence is a nonlinear function of fractional NADH saturation (α) when $K_d = 4.2 \times 10^{-6}$ M is assumed (Figure 2), in accord with the results of Stinson & Holbrook (1973). To remove the nonlinearity, an unacceptably low

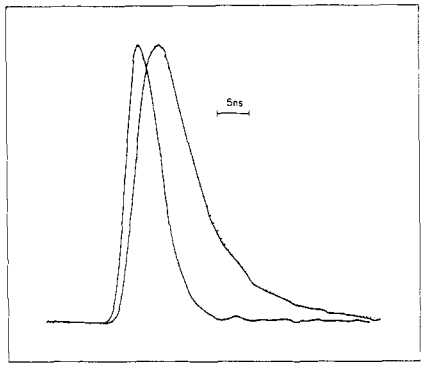

FIGURE 1: Excitation and fluorescence profiles of LDH (—); convolutions of $I(t) = 0.5e^{-t/1.0} + 0.16e^{-t/14.0} + 0.37e^{-t/7.4}$ and $I(t) = 0.53e^{-t/0.94} + 0.47e^{-t/6.79}$ are indistinguishable and represented by (···).

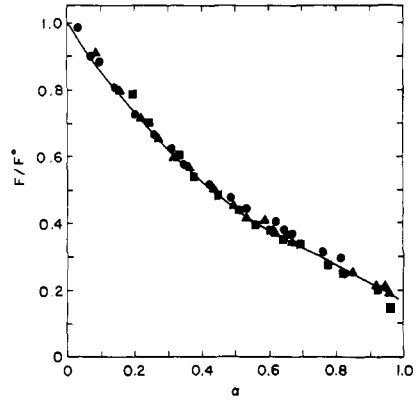

FIGURE 2: Quenching of LDH emission (295-nm excitation) by NADH with α calculated from $K_d = 4.2 \times 10^{-6}$. The several symbols (●, ▲, ■) designate measurements recorded on different days. [LDH] = 1.8×10^{-6} M.

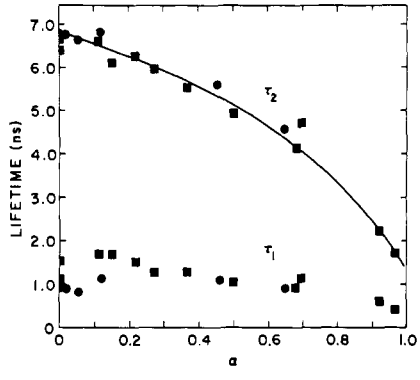

FIGURE 3: LDH lifetimes from a two-component least-squares fit as a function of fractional saturation with NADH (α calculated from $K_d = 4.2 \times 10^{-6}$). ● and ■ designate results from different data sets. [LDH] = 1.8×10^{-6} M.

value, $K_d = 2 \times 10^{-6}$ M, must be used to calculate the fraction complexed. Thus, we confirm the nonlinear quenching behavior described by Holbrook.

The decay-function parameters were obtained from two-component fits. In order to minimize the long-term parameter variations described above, lifetime measurements were completed in 1 day on a complete series of LDH samples with different NADH concentrations. The lifetimes from two such data sets are plotted in Figure 3 as a function of fractional saturation with NADH. The τ_2 values for the two sets are in good concordance, but τ_1 exhibits scatter. However, $a_1\tau_1$ is constant, and we do not believe that τ_1 varies appreciably with NADH concentration, at least to $\alpha = 0.7$. The reduction in τ_1 for $\alpha > 0.9$ may be real. The preexponential factors, a_1

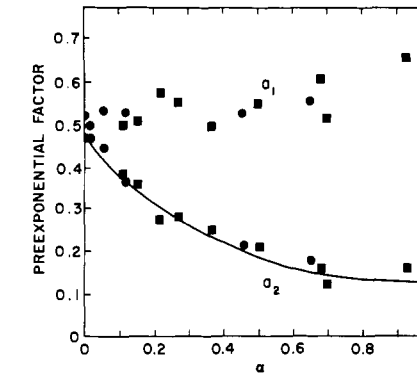

FIGURE 4: Preexponential factors from a two-component least-squares analysis of LDH emission for varied fractions of NADH binding (α, calculated from $K_d = 4.2 \times 10^{-6}$). ● and ■ correspond to the same data sets as in Figure 3. [LDH] = 1.8×10^{-6} M.

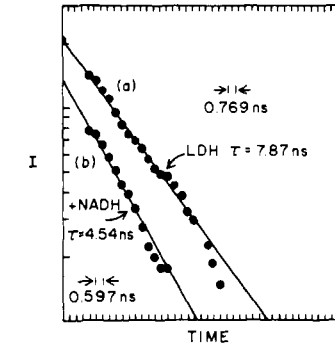

FIGURE 5: Semilog plots of the long-lived decay: (a) LDH alone (2.06×10^{-6} M), (b) LDH + (1.15×10^{-5}) M NADH ($\alpha = 0.71$). Note difference in time scales.

and a_2 (Figure 4), were scaled by using the steady-state fluorescence intensities, i.e.,

$$\frac{F}{F^0} = \frac{a_1\tau_1 + a_2\tau_2}{a_1^0\tau_1^0 + a_2^0\tau_2^0}$$

subject to the requirement that a_1/a_2 be invariant to the scaling procedure and that $a_1^0 + a_2^0 = 1$. The superscript refers to zero NADH concentration.

Long-Lived Decay Components. The decay profiles were examined at long times after excitation (>40 ns). This was accomplished by changing the time base for the measurements. The excitation pulse has no contribution in this region, and linear semilog plots could be obtained (Figure 5), leading to lifetimes in the range 7.4–8.1 ns, substantially longer than τ_2 obtained from the two-component least-squares fit. Moreover, when 1.16×10^{-5} M NADH was added, the "tail" lifetime was reduced to 4.6 ns, in good agreement with $\tau_2 = 4.6$ ns computed for the same sample by least-squares fitting. We do not ascribe any importance to this agreement except to conclude that a slow response time of the detection system (Demas & Crosby, 1970) is not the source of the 7.4-ns tail component. Evidently, the value of τ_2 extracted from the data using a two-component model is an average of several decays.

Discussion

There are six tryptophans in each of the four identical subunits of pig heart LDH. If all the tryptophans have the same radiative rate constant (Ricci, 1970), it is possible to estimate the fraction (f) that emits with sufficient intensity to be recorded (Hazen et al., 1976),

$$f = \frac{q\tau_r}{\sum a_i\tau_i}, \qquad \sum a_i = 1 \tag{2}$$

where τ_r is the lifetime of a reference molecule (*N*-acetyl-

Table I

residue no.	R(A), Trp-NADH separation[a]	$1/R^6 \times 10^8$	exposure[b]
150	19.9	1.61	intermediate
190	19.7	1.71	internal
203	23.5	0.59	internal
225	16.5 (>30)[c]	4.96 (<0.14)	internal
248	13.1	19.8	internal
323	21.7	0.96	external

[a] From Holbrook et al. (1975); numbering according to sequence of Eventoff et al. (1977). [b] For dog-fish LDH (Eventoff et al., 1977). [c] Estimated from residue-231 coordinates.

tryptophanamide) and q is the quantum yield of LDH relative to this reference. A two-term summation with $a_1 + a_2 = 1$ is a good approximation if a good fit to the experimental decay is obtained with such a function (Hazen et al., 1976). The relative quantum yields, measured for several excitation wavelengths in the range 290–305 nm, where tyrosine absorption is small, were between 1.42 and 1.50. These quantum yields lead to $f = 1.06$, i.e., all six tryptophans of each subunit emit.

The contribution of all six tryptophans to the emission requires, in principal, a six-term decay function, $I(t) = \sum_{i=1}^{6} a_i e^{-t/\tau_i}$, to describe the pulsed excitation behavior. The data quality does not justify the use of more than two terms in the least-squares fitting procedure. The uncertainties inherent in this procedure will be described below.

NADH Quenching. Steady-state quenching measurements do not provide a basis for a detailed analysis of the NADH quenching. The pulsed excitation results are, however, helpful in such an analysis. The following are the most pertinent points describing the effect of NADH binding on τ_1, τ_2, a_1, and a_2 (Figures 3 and 4). (1) a_2 is reduced upon NADH addition, and the limiting value for the fully complexed enzyme is $a_2^{\infty} = {}^1/_3 a_2^0$. The major decrease in a_2 occurs before the point of 50% saturation. The long-lived components represented by a_2 are responsible for 85% of the steady-state emission ($a_2^0\tau_2^0 \approx 6a_1^0\tau_1^0$). (2) τ_2 also decreases with NADH binding to a limiting value of ~1.7 ns for the fully complexed enzyme. In contrast to the behavior of a_2, the largest τ_2 decrease takes place after 50% saturation. (3) Neither a_1 nor τ_1 changes very much with NADH binding. The following paragraphs provide a basis for understanding these observations. It should be noted that it is the changes in τ_1, τ_2, and a_1/a_2 that are of major interest, and our conclusions are not dependent upon the absolute values of the parameters.

Since bound NAD^+ does not quench the LDH emission (Stinson & Holbrook, 1973) and the tryptophan emission and NADH absorption spectra overlap markedly, the quenching is presumed to occur via resonance transfer (Förster, 1959) with rate constants given by

$$k_{\mathrm{Trp}\rightarrow\mathrm{NADH}} = \text{constant}\frac{\kappa^2}{R^6} \qquad (3)$$

where R is the NADH–Trp separation in dogfish LDH (Table I) and κ^2 is related to the mutual orientation of the donor and acceptor transition moments. If the quenching rate is so large that emission from a quenched tryptophan is below the measurement noise level, we call it strong quenching. A reduction in a_i is the criterion for strong quenching. Less efficient quenching will reduce τ_i without affecting a_i, and this is termed moderate quenching. In terms of this criterion, the diminution of a_2 is the result of strong quenching and the residual a_2 implies that some of the long-lived emission is only moderately quenched. If the quenching of τ_2 reduced the lifetime to ~1 ns, this would be evidenced by an increase in a_1, contrary to observation.

Table II: Fitting of Three-Component Simulations with Two-Component Functions

	assumed parameters						two-term results[a]				
	a_1'	τ_1'	a_2'	τ_2'	a_3'	τ_3'	a_1	τ_1	a_2	τ_2	$\langle\tau_2\rangle$[b]
(i)	0.5	1.0	0.16	4.0	0.34	8.0	0.544	1.15	0.456	7.18	6.72
(ii)	0.5	1.0	0.34	4.0	0.16	8.0	0.579	1.23	0.421	5.86	5.28
(iii)	0.5	1.0	0.16	4.0	0.34	7.4	0.546	1.13	0.460	6.68	6.30
(iv)	0.5	0.5	0.25	4.0	0.25	7.4	0.502	0.715	0.498	6.05	5.70

[a] From least-squares fit of convoluted three-term function. [b] $\langle\tau_2\rangle = (a_2'\tau_2' + a_3'\tau_3')/(a_2' + a_3')$.

Analysis of Time-Resolved Fluorescence Measurements. The incompleteness of a two-term representation of the decay data has been emphasized already. The NADH quenching results indicate that about half of the residues are short-lived (ca. <1 ns) and not quenched; we place three residues in this unquenched category. Of the remaining three residues one and perhaps two have lifetimes exceeding 7 ns and the other residue(s) a lifetime between 1 and 7 ns. As shown in Figure 1, $I(t) = 0.5e^{-t/1.0} + 0.16e^{-t/4.0} + 0.34e^{-t/7.4}$ fits well the experimental decay. This function is consistent with $a_2^0/a_1^0 \approx 1$, with $a_2^{\infty} = {}^1/_3 a_2^0$ and with two components averaged into τ_2. However, an equally good fit results from other parameter choices, e.g., $I(t) = 0.5e^{-t/0.5} + 0.25e^{-t/4.0} + 0.25e^{-t/8.0}$. Due to the correlation between a_i and τ_i, an infinite number of parameter sets will yield a satisfactory fit. In view of the NADH quenching results and the tail lifetime, we assign, subject to a caveat described below, each tryptophan residue to one of three lifetime classes, viz., 1, 4, and 8 ns. These numbers are used to classify and should not be taken literally. Indeed, for the model described, the data give 7.4 ns as the lifetime of a residue in the 8-ns class.

A fundamental question that arises when emission from three or more residues is fit with a two-term function in a least-squares analysis is—how are the various "real" components mixed in the fitting to obtain a smaller number of averaged parameters? To provide a guide, we convoluted several three-term functions with an excitation pulse and analyzed these simulations with the two-term fitting program using the same excitation pulse. The pertinent results are summarized in Table II. First, these simulations suggest that most of τ_2', the 4.0-ns component of the three-term function, as well as τ_3', is included in τ_2, whereas τ_1 is nearly "pure" 1.0-ns τ_1'.

Secondly, the weighting in the averaging of the real components should be considered. The fluorescence intensity used in eq 2 is $\sum a_i\tau_i$ with $\sum a_i = 1$. A naive weighting procedure is

$$\langle\tau_2\rangle = \frac{a_2'\tau_2' + a_3'\tau_3'}{a_2' + a_3'}$$

This assumes that the total emission characterized by τ_2 is represented by $\langle\tau_2\rangle$, which in turn consists of the contributions $a_2'\tau_2'/(a_2' + a_3')$ and $a_3'\tau_3'/(a_2' + a_3')$. For example, the calculated value, $\langle\tau_2\rangle = [(0.16 \times 4.0) + (0.34 \times 8.0)]/0.5 = 6.72$ ns, compares well with τ_2 (7.18 ns) obtained from the least-squares fitting of the three-term function ($a_1' = 0.5$, $\tau_1' = 1.0$, $a_2' = 0.16$, $\tau_2' = 4.0$, $a_3' = 0.34$, $\tau_3' = 7.4$) by a two-term decay (Table II). Although the above simple weighting procedure leads to values of $\langle\tau_2\rangle$ that are slightly low, we will use $\langle\tau_2\rangle$ in the evaluation of models.

Thirdly, when the number of emitters exceeds the number

of terms in the fitting function, there is an additional problem, viz., the parameter values depend on the number of points (the time period) used in the analysis. This is evident even when noise-free simulations are tested. A two-term fit to a two-term simulation function yields precisely the input parameters, irrespective of the number of input data points employed. A two-term fit to a three-term simulation function yields values of τ_1 and τ_2 that decrease progressively as the number of data points is reduced. In view of this, we subjected the experimental data to analysis with different numbers of data points. None of the trends in τ_1 and τ_2 were altered. These points serve to underscore the difficulties that are encountered in the determination of absolute fluorescence decay data when multiple emission prevails.

To summarize, the pulsed excitation results for free LDH are fit well by the three-component set of parameters (iii) of Table II, in which two residues have long lifetimes (near 8 ns), one has a moderately long lifetime (near 4 ns), and three are of short lifetime (near 1 ns). Binding of NADH quenches strongly two and moderately one of the three longest lifetime emitters. Although other models are consistent with the data, the above model is plausible, indeed, in our view, the most plausible. In any case, by restricting our analysis to changes in a_i and τ_i, we believe that physically meaningful conclusions can be extracted from the quenching data.

Assignment of the Tryptophan Emission. In order to interpret the experimental results in terms of emission from individual tryptophan residues, two kinds of information must be obtained from the three-dimensional structures of the free enzyme and the NADH–enzyme complex. These are Trp–NADH separations, to infer the Trp → NADH energy transfer, and exposure to solvent, to interpret the I^- and other small-solute quenching results (vide infra). The published Trp–NADH distances (Holbrook et al., 1975), given in Table I, are based on a crystallographic determination that has been revised in some important details. The positions of Trp-150, -190, -203, and -323 have not been altered, and the Table I interchromophore distances for these residues are valid. Trp-249 of Holbrook et al. (1975) has become, with renumbering of the sequence, residue 248. The most striking change involves Trp-225, for which the revised intrasubunit Trp-225–NADH distance exceeds 30 Å. With allowance for these changes, the τ_2 and a_2 variation with NADH binding can be interpreted. The fluorescence properties of LDH fully complexed with NADH (in particular $a_2^\infty < a_2^0$) require that at least one and probably two long-lived tryptophan residues be strongly quenched. The nonlinear decrease in a_2 with increase in α requires that a strongly quenched residue be subject to intersubunit quenching. Trp-248 is the only residue that can be subject to intersubunit quenching (R = 16.2 Å) (Holbrook et al., 1975). All other intersubunit distances exceed 33 Å. The nonlinear decrease in a_2 means that Trp-248 is one of the strongly quenched residues. The relatively slow decrease in τ_2 with α can be understood similarly. In our model, τ_2 is composed of contributions from the unquenched part of the emission of one or, more likely, two strongly quenched residues, as well as the emission, quenched and unquenched, from the moderately quenched residues(s). Because of the weighting involved ($\langle\tau_2\rangle = \sum a_i\tau_i/\sum a_i$), any reduction in $\sum a_i$ with binding will lead to a nonlinear variation in $\langle\tau_2\rangle$. In particular, a_2 will decrease at a greater rate than τ_2 (vide infra).

Support for the foregoing interpretation comes from the following calculation. Binding of a single NADH quenches two Trp-248 residues (one in the complexed subunit and another in an adjacent subunit). When two NADH are bound, an average of 3.3 Trp-248 chromophores are quenched. In species with three or four NADH all the Trp-248 emission is quenched. At 50% saturation, the fractions of each species are EL_0 = 0.035, EL_1 = 0.184, EL_2 = 0.362, and $EL_3 + EL_4$ = 0.419 (Holbrook, 1972). Under these conditions only 25% of the Trp-248 will emit (8 ns), but 50% of the remaining 8- and 4-ns emission will persist. One of these latter residues is moderately quenched ($\tau_2 \approx 2$ ns). We consider now two possibilities. If both of the 8-ns residues are strongly quenched, $\langle\tau_2\rangle$ = 5.0 ns. If Trp-248 and the 4-ns residue are strongly quenched, $\langle\tau_2\rangle$ = 4.9 ns. Each of these values is in good agreement with the least-squares fit (τ_2 = 5 ns at half-saturation with NADH) and indicates the origin of the more rapid decrease in a_2 compared to τ_2. This accord may be fortuitous, but the proposed model for the fluorescence of the free protein is plausible.

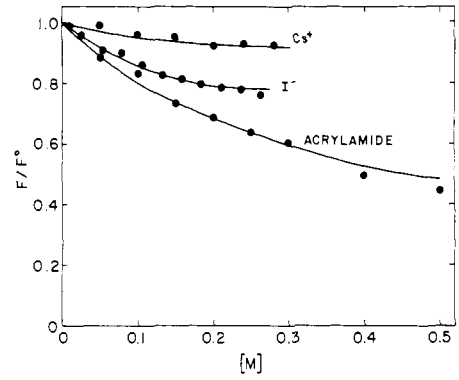

FIGURE 6: Quenching of LDH steady-state emission by small molecules. LDH concentrations were 4.4 × 10^{-7} M for I^- and acrylamide and 6.4 × 10^{-7} M for Cs^+.

Although the identification of Trp-248 as a long-lived (8 ns) residue strongly quenched by NADH can be made with some confidence, the assignments of the other emitters are less certain. Energy-transfer arguments are helpful in this connection. In order to use eq 3 in a quantitative fashion, the κ^2 factors should be evaluated. A visual examination of a stereoscopic projection (Holbrook et al., 1975) indicates that the π–π^* transition moments of nicotinamide are neither coplanar nor perpendicular to any of the π–π^* tryptophan transition moments, and there is no reason to expect widely different values of κ^2.

Quenching by I^-, Cs^+, and Acrylamide. The quenching of LDH emission by I^-, Cs^+, and acrylamide is shown in Figure 6. Neither τ_2 nor τ_1 is sensibly affected by 0.24 M I^-. In contrast, both τ_1 and τ_2 are reduced and a_2 is diminished by 40% in 0.30 M acrylamide solution.

The bimolecular constants for the quenching of *N*-acetyltryptophanamide by I^- and Cs^+ are 2.6 × 10^9 and 1.5 × 10^9 M^{-1} s^{-1}, respectively (Shinitzky & Rionay, 1977). The smaller LDH quenching by Cs^+ is thus not unexpected. In any event, the relatively poor quenching efficiency of LDH emission by I^- is not due to charge effects.

Only one of the LDH tryptophans is highly exposed to solvent (Table I). Rapid conformational fluctuations do not appear to make the buried residues accessible to I^-. The Stern–Volmer plot for acrylamide quenching curves upward, which indicates static quenching (Eftink & Ghiron, 1976). The reduction in $\sum a_i$ induced by acrylamide is further evidence for static quenching. The greater penetrating power of acrylamide is more likely due to an equilibrium rather than a diffusion effect, since the quenching is at least partly static. The ability of acrylamide to quench buried tryptophans has

Table III: Relative Activities of LDH Solutions

	rel. act.[a] (%)
LDH, $\mu = 0.3$	100.0
LDH, $\mu = 1.02$	74.2
I^-, 0.28 M ($\mu = 0.3$)	74.5
I^-, 0.4 M ($\mu = 1.02$)	4.7
I^-, 1.0 M ($\mu = 1.02$)	3.0
Cs^+, 0.28 M ($\mu = 0.3$)	83.5
acrylamide, 0.3 M ($\mu = 0.3$)	81.6

[a] Relative to value at $\mu = 0.3$. Reaction conditions: [NADH] = 1.55×10^{-4} M, [pyruvate] = 3.6×10^{-4} M, [LDH] = 1.66×10^{-9} M. 0.02 M Tris buffer (pH 7.4). Ionic strengths were adjusted with NaCl.

Table IV: Tryptophan Lifetimes and Quenching Sensitivity

Trp	lifetime class	NADH quenching	I^- quenching
150	? 8	high	moderate
190	? 4	moderate	? slight
203	1	slight	slight
225	1	slight	slight
248	8	high	slight
323	1	slight	highest

been documented (Eftink & Ghiron, 1975, 1976).

Since only 15% of the emission can be ascribed to the 1-ns unquenchable class of emitters and I^- quenches at least 25% of the emission, a long-lived residue must be I^- quenchable. Trp-150 is the best candidate for this emitter.

When the I^- concentration is increased to 0.4 M, there is a decrease in activity (Table III). Apparently I^- binding is not to be neglected.

The distance argument clearly consigns Trp-225 to the short-lived nonquenchable class, and Trp-203 and -323 probably belong in this category. This leaves Trp-150 and -190 to be assigned. One of these is highly quenched and the other moderately quenched by NADH. One is in the 8-ns lifetime class, the other is in the 4-ns class, but the Trp–NADH separations alone do not permit any further inferences. The I^- quenching data discussed above suggest that Trp-150 is the longer lived chromophore and hence more readily quenched by NADH.

Tryptophan Environment and Lifetime. The λ_{max} (337 nm) of the fluorescence spectrum and the exposure estimates given in Table I indicate that none of the emitters are highly exposed to solvent. Yet, the lifetimes vary considerably. Clearly, lifetime alone cannot be used to distinguish buried from exposed residues. A similar situation is encountered in lysozyme where no correlation between exposure and lifetime prevails (Formoso & Forster, 1975).

A more detailed analysis of the effect of tryptophan environment on the lifetime must await the availability of the three-dimensional structure of pig heart LDH.

Conclusions

The pertinent conclusions are contained in Table IV. The most important points are as follows. (1) All six tryptophans in each subunit emit, and there are at least three lifetime classes. (2) Bound NADH quenches some tryptophans very strongly, others only moderately, and some not measurably. The short-lived residues (ca. <1 ns) are in the last class. (3) Nonlinear quenching by NADH is due to intersubunit quenching of Trp-248.

If the donor–acceptor distance is the dominant factor in Trp → NADH energy transfer and if solvent exposure calculations are indicative of accessibility to I^- quenching, a reasonable assignment of the residues can be made (Table IV), but some ambiguities remain. We emphasize that the detailed assignments must be viewed with some caution. For example, the model assumes $a_2/a_1 = 1$, but the experimental values of a_2/a_1 were between $^2/_3$ and 1. Nonetheless, it is clear that the analysis of multiple chromophore emission with a two-term decay function can lead to meaningful conclusions. A two-component analysis of the decay results alone is not sufficient, and auxilliary information, such as quenching by small solutes or analysis of the long-time decay, is needed. The analysis made in this work is independent of the crystal structure results, except for the assignment of fluorescence properties to particular residues.

In order to use fluorescence results in formulating proposals about enzyme mechanisms and conformation it is necessary to have a more complete understanding of the characteristics of each emitter than is usually available from steady-state measurements. For example, quenching of tryptophan and tyrosine emission by small molecules is widely used to assess the accessibility of emitters to these quenchers. However, the steady-state quenching constants are functions of the chromophore lifetime as well as its exposure to solvent. When more than one emitter is present, these contributions of the individual residues to the single constant cannot easily be detailed (Lehrer, 1976).

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