Lifetimes and NADH Quenching of Tryptophan Fluorescence in Pig Heart Cytoplasmic Malate Dehydrogenase*

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The time-resolved and steady state fluorescence properties were measured for pig heart cytoplasmic malate dehydrogenase at pH 6.0 and 8.0. The fluorescence decay can be described by two rate processes, according to the functions: $I(t) = 0.7e^{-t/18} + 0.3e^{-t/4.2}$ for the free enzyme and $I(t) = 0.7e^{-t/0.8} + 0.3e^{-t/28}$ for the enzyme-NADH complex. Quenching by NADH of the tryptophan fluorescence is linear. The only effect of pH is to change the association constant for NADH binding; the fluorescence of the free enzyme and the fluorescence quenching by NADH, I, and acrylamide are unaffected by pH. Thus there are no changes in conformation of the free enzyme or of the NADH complex over the range of pH 6 to 8.

Structural homology between dehydrogenases has been established (1). Particularly evident are the structural similarities between the subunits in cytoplasmic malate dehydrogenase (s-MDH)* and lactate dehydrogenase (LDH). Therefore, it is of interest to describe and to compare the physical properties of these two proteins, with the expectation that analysis of the differences is an effective route to understanding the structural basis of the enzyme properties.

Each subunit of the H4 isozyme of pig heart LDH contains 6 tryptophans. The sequence of pig heart cytoplasmic MDH has not been reported, but tryptophan analysis has indicated 5 residues in each subunit of the dimeric enzyme (2). Binding of NADH results in quenching of the fluorescence of both LDH and s-MDH. For s-MDH the fluorescence decreases linearly with fractional saturation by NADH, which is in marked contrast to the nonlinear quenching of the tryptophan emission of LDH.

Considerable discussion has centered on the equivalence of the two NADH binding sites of s-MDH. Positive cooperativity in NADH binding has been reported for beef heart s-MDH (3), and negative cooperativity for the pig heart enzyme under certain conditions (4). Recent measurements of NADH fluorescence enhancement have shown that the binding sites are independent in the pig heart enzyme (5).

The tryptophan emission in LDH has been studied by a time-resolved method (6), and three classes of tryptophans were distinguished according to their decay times. Three of the six tryptophans of LDII were not quenched by bound NADH and had short decay times (~1 ns). The emission from the other 3 tryptophans was quenched by Trp → NADH energy transfer, and these chromophores had lifetimes in the range 4 to 8 ns. In the free enzyme one of these chromophores has an 8-ns lifetime, and two have 4- to 5-ns lifetimes. Quenching by NADH reduces the lifetimes to 2 ns or less.

The studies reported in this work define the time-resolved fluorescence properties of s-MDH, in order to examine the extent to which structural and enzymological homology is reflected in fluorescence properties. A principal conclusion from this work is that the fluorescence emissions of LDH and s-MDH are similar, and that, in particular, both the linear NADH quenching of s-MDH and the nonlinear NADH quenching of LDH emission are due to energy transfer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pig heart cytoplasmic malate dehydrogenase (s-MDH) was prepared by the method of Glatthaar et al. (7), but the acid precipitation and CM-cellulose steps were omitted, and chromatography on hydroxylapatite (8) was added as a final step. The first two of the three peaks of activity eluted from the hydroxylapatite column were used. All s-MDH solutions were treated with charcoal in 0.3 M NaCl. The purified NADH stock solutions had an absorbance ratio of 2.25 to 2.28. Concentrations of NADH were determined using ε280 = 7.2 × 104 M⁻¹ cm⁻¹, measured in this laboratory.

**Steady State Fluorescence Measurements**—Fluorescence intensities were measured with an Aminco-Bowman Spectrofluorometer thermostatted at 26.0 ± 0.1°C. NADH emission was excited at 340 nm and monitored at 450 nm, and tryptophan emission was excited at 285 nm and monitored at 340 nm. Corrections for inner filter effects and fluorescence reabsorption have been described in detail (6). Briefly, for NADH emission the measured fluorescence intensity is multiplied by 10^{AF} ≈ A = absorbance (340 nm) to correct for inner filtering. For tryptophan emission, an empirical correction factor was determined by measuring the fluorescence intensity of N-acetyltryptophanamide as a function of NADH concentration. Excitation spectra were determined at 25 ± 1°C with a modular spectrofluorometer assembled in this laboratory.

**Binding constants** were determined by assuming that the fractional NADH binding, α, is proportional to ΔF, the fluorescence enhancement of bound NADH or the fluorescence quenching of tryptophan:

$$\alpha = \frac{\Delta F}{\Delta F_{\text{max}}}$$
The dissociation constant for the reaction $EL = E + L$ is then:

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

where $[L]_0$ and $[E]_0$ are the total NADH and enzyme concentrations, respectively, $\Delta F_{ext}$ and $K_d$ are treated as free parameters in a least squares fitting procedure.

Lifetime Measurements—Tryptophan fluorescence was excited at 295 nm with an ADP-doubled Rhodamine 6G dye laser, pumped with an Nd$_3^+$ laser (10). Measurements were made at room temperature (24ºC). Lifetimes were obtained by convoluting a two-term decay function, $I(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$, with the excitation pulse, using nonlinear least squares (trended Taylor series or Simplex) fitting routines. Changes in $a_1$ and $a_2$ induced by NADH binding were calculated by scaling $\alpha_1a_2$ to the steady state fluorescence intensities.

For further discussion of the fluorescence measurements and data reduction, see Ref. 6.

RESULTS AND DISCUSSION

Fluorescence Lifetimes of Free s-MDH—The uncertainties encountered in extracting $a_1$ and $\tau_1$ from the experimental excitation and fluorescence intensity profiles, using a two-term decay function with a least squares fitting procedure, have been discussed by Torikata et al. (6) with regard to work with LDH. The important points are as follows: (i) The absolute values of $\tau_1$ and $a_1$ vary somewhat from day to day. The extremes of the range for s-MDH at pH 8.0 were $I(t) = 0.85e^{-t/0.88} + 0.35e^{-t/4.98}$ and $I(t) = 0.68e^{-t/1.02} + 0.32e^{-t/4.41}$. A similar variation was found at pH 6.0. (ii) The precision of the data for several runs with one sample was typically: $\tau_1$ 0.2 ns; $\tau_2$ 0.1 ns; $a_1$, 0.1; $a_2$, 0.03. The products $a_1\tau_1$ tend to remain constant, i.e. an increase in $a_1$ is accompanied by a compensating decrease in $\tau_1$. (iii) The best fit values of $\tau_1$ and $a_1$ depend on the time span of the data chosen for analysis. In this regard, it is physically reasonable to work with time periods long enough to include all the fluorescence intensities that are above the noise level, and the results described below are based on this choice.

In view of the results obtained with LDH (6), which were summarized above, it is appropriate to describe the results with s-MDH in terms of a qualitative comparison between the fluorescence decays of s-MDH and LDH. The salient points are as follows: (i) The long lived (~8 ns) component found in LDH is absent in s-MDH. (ii) The ratio $a_2/a_1$ is 1/2 for s-MDH and is to be compared to a near-unity value for LDH, i.e. the contribution from the short lived residues is proportionately larger for s-MDH. (iii) The dependence of $\tau_1$ on the time interval used in the data analysis is analogous to the situation that was found for LDH. As the point of termination of data collection is changed from the time where the fluorescence intensity reaches the noise level to shorter times, $\tau_2$ decreases. Since the same trend is found in analyzing a three-component noise-free simulation with a two-term least squares fitting program, we believe that this behavior indicates that there are at least three decay components in the MDH emission.

Quenching of Steady State Fluorescence by NADH—As NADH is added to s-MDH, the tryptophan emission is progressively quenched, and the NADH emission intensity increases concomitantly (6). The enhanced NADH emission provides an excellent tool for determining the dissociation constant for the protein-NADH complex. Our values of $K_d$ (Table I) agree well with those determined by Lodola et al. (5), namely, $0.6 \times 10^{-4}$ M in 0.05 M phosphate buffer of pH 6.0 and $11 \times 10^{-4}$ M in 0.5 M Tris buffer of pH 8.0. Lodola et al. (5) estimated $\Delta F_{ext}$ from the recorder trace and used a method similar to ours to correct for inner filter effects. The NADH fluorescence thus is a linear function of the fractional satura-

<p>| TABLE I |
| Dissociation constants of the NADH-MDH complex at 26°C, measured by the dependence of fluorescence on NADH concentration |</p>
<table>
<thead>
<tr>
<th>Fluorescence measurement</th>
<th>pH 6.0</th>
<th>pH 8.0</th>
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<tbody>
<tr>
<td>0.05 M phosphate</td>
<td>$\mu = 0.3$</td>
<td>$\mu = 0.3$</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>0.71 x $10^{-4}$</td>
<td>6.7 x $10^{-4}$</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>0.80 x $10^{-4}$</td>
<td>5.7 x $10^{-4}$</td>
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</table>

tion. We confirm the observation of Lodola et al. (5) that s-MDH tryptophan emission likewise varies linearly with fractional saturation (Fig. 1). Consequently, $K_d$ can also be computed from tryptophan quenching by NADH and the agreement with the values of $K_d$ from fluorescence enhancement of NADH is excellent (Table I).

Although $K_d$ for binding of NADH changes nearly 10-fold between pH 6.0 and 8.0, the effect of bound NADH on tryptophan emission is essentially the same at pH 6.0 and 8.0. Fig. 1 shows a superposition of data for these two pH values. This result is noteworthy because it rules out conformation changes affecting tryptophan environment in the pH range 6.0 to 8.0. Furthermore, because NADH quenching is by energy transfer that is sensitive to geometry, there can be no change in the NADH complex between pH 6.0 and 8.0.

Phosphate binds to LDH at pH 6.0 and quenches the tryptophan emission. Neither the steady state intensity nor the lifetimes of tryptophan in s-MDH are affected by phosphate in the concentration range 0.05 to 0.2 M.

NADH Quenching Mechanism—In contrasting the nonlinear quenching of LDH tryptophan emission by NADH to the linear quenching of the s-MDH emission, Lodola et al. (6) concluded that linear quenching cannot result from Förster resonance energy transfer. Instead, they postulated a conformation change induced by NADH binding.

The following experiments show that Trp → NADH energy transfer suffices to explain NADH quenching of s-MDH fluorescence. Fig. 2 gives excitation spectra of LDH and s-MDH with bound NADH monitored at 450 nm. The 290 nm band in the excitation spectra of LDH-NADH and s-MDH-NADH is absent in the excitation spectrum of NADH alone (Fig. 2). The protein and NADH concentrations were chosen so that 50% of the total NADH was formed in a complex. Under these conditions the emission from free NADH is relatively small. The NADH emission is directly excited at 340 nm, but at 280 nm 90% of the exciting light is absorbed by the protein, and the intense NADH emission can only result from Trp → NADH energy transfer.

The NADH fluorescence quantum yields for excitation at 340 nm and 280 nm are related by the following equation:

$$\Phi_{280} = \Phi_{340} [1 - f] + f \cdot \epsilon$$

where $\epsilon$ is the fraction of light absorbed by the protein and $f$ is the efficiency of energy transfer. The first term within the brackets represents fluorescence of NADH directly excited by light of 280 nm wavelength, and the second term corresponds to NADH fluorescence induced by energy transfer from tryptophan.

Under the conditions of Fig. 2, $f = 0.92$ for LDH and 0.91 for s-MDH. The energy transfer efficiencies for the two proteins are related by:

$$\frac{0.92_{s-MDH} + 0.09}{0.92_{s-MDH} + 0.09} = \frac{6 \cdot (F_{280}/F_{340})_{s-MDH} - 0.92_{s-MDH} + 0.08}{5 \cdot (F_{280}/F_{340})_{s-MDH} - 0.92_{s-MDH} + 0.08}$$

where $F_{280}$ and $F_{340}$ are the intensities (corrected for inner
Filter effects of NADH emission excited at 280 and 340 nm, respectively. The factor 6/5 is the ratio of the number of tryptophans in LDH and s-MDH. Application of Equation 1 gives a value of $e_{\text{MDH}}/e_{\text{LDH}}$ between 0.65 and 0.69. If energy transfer were entirely responsible for tryptophan quenching in both LDH and s-MDH, this ratio should correspond to the steady state quenching ratio at $Y = 1$, which is 0.45/0.85 = 0.52. Considering the uncertainty in the inner filter corrections, the agreement between these two ratios is satisfactory, and therefore, one can conclude that the energy transfer efficiencies in the two proteins are comparable.

Equation 1 can be used to calculate the absolute transfer efficiency, since

$$F_\text{340} = \frac{(I_{\text{340}})_{\text{280}}}{(I_{\text{280}})_{\text{340}}} \left[ (1 - f) + f \epsilon \right]$$

where $A_{280}$ and $A_{340}$ are the absorbances of the complex at the two wave lengths. $I_{340}/I_{280}$ was estimated by means of a Rhodamine B quantum counter. Application of Equation 2 to LDH yields $\epsilon = 1.01$, which is to be compared with 0.85 from the steady state quenching in the complex (6). We conclude that most, if not all, of the tryptophan quenching by NADH is due to Trp → NADH resonance energy transfer. This conclusion holds for s-MDH also, because we have shown above that $e_{\text{LDH}}/e_{\text{MDH}}$ corresponds to the ratio of the quenching fractions at saturation.

The Effect of NADH on Fluorescence Lifetimes—Figs. 3 and 4 give for pH 8 and pH 6, respectively, the values of the parameters $\tau_1$ and $\alpha_1$ from the least squares fitting of pulsed excitation results for s-MDH, as a function of the fractional saturation with NADH. To minimize variations due to instrument settings, sample preparation, and artifacts of data analysis, a data series covering the full range of saturation was recorded on a single day. Two such series are included in the pH 6.0 results, and three series are included for pH 8.0. The following points merit emphasis: (i) There is little, if any, difference between the decay parameters obtained for pH 6 and pH 8. (ii) $\tau_2$ decreases linearly with $\alpha$. (iii) The limiting value of $\tau_2$ (at full NADH saturation) is 2 ns. (iv) $\tau_1$ is independent of $\alpha$ at both pH values. (v) Neither $\alpha_2$ nor $\alpha_1$ vary significantly with $\alpha$.

The contrast between the effect of NADH on $\alpha_2$ and $\tau_2$ of s-MDH and LDH is striking. With LDH, $\alpha_2$ decreases with the binding of NADH, indicative of strong quenching, i.e. reduction of some of the long lived emission below the detection level. Strong quenching is absent in s-MDH. Also, the nonlinear dependence of $\tau_2$ on $\alpha$ for LDH is associated with the reduction in $\alpha_2$ and is not found for s-MDH.

Quenching by Acrylamide and $\Gamma$—Fig. 5 gives the results of steady state measurements of quenching by acrylamide and $\Gamma$ ion. The quenching efficiencies of both $\Gamma$ and acrylamide are each the same at pH 6.0 and 8.0. Approximately 15% of the fluorescence is quenched in 0.23 M $\Gamma$. Time-resolved measurements show that $\tau_2$ is also reduced 15% and $\alpha_2/\alpha_1$ is unchanged. Acrylamide is a much better quencher than $\Gamma$ and nearly 80% of the emission is acrylamide-quenchable. Pulsed measurements made for 0.3 M acrylamide solutions show that neither $\Sigma\alpha_i$ nor $\alpha_2/\alpha_1$ were changed by the acrylamide. This observation is in marked contrast to that for LDH, for which both $\alpha_2/\alpha_1$ and $\Sigma\alpha_i$ decreased (6). Any change in $\Sigma\alpha_i$ is interpreted as static quenching; the invariance of $\Sigma\alpha_i$ for s-MDH points to a dynamic quenching mechanism. Both long and short lived residues in s-MDH are accessible to acrylamide.

Comparison of s-MDH and LDH—Some similarities and differences in the fluorescence behavior of s-MDH and LDH have been described. These and other comparisons are summarized in Table II. Because the sequence of s-MDH is not available, discussion in terms of identifiable tryptophans is speculative. Nonetheless, some significant issues can be raised.

The most striking differences between s-MDH and LDH are (i) the absence of a long lived (8 ns) component in s-MDH and (ii) the linear quenching by NADH found for s-MDH. In LDH, the nonlinear quenching is due to the close proximity of Trp-248 to two NADH binding sites, one within the subunit and one in an adjacent subunit (6). Trp-248 was assigned an 8-ns lifetime. There are 6 tryptophans in each LDH subunit but only 5 in the s-MDH subunit. The simplest interpretation of the difference in NADH quenching is that the analog of Trp-248 of s-MDH is not present in a MDH. Alternatively, if the s-MDH residue sequentially equivalent to LDH Trp-248 is present, it must be in a different environment, to explain the shorter lifetime. In order to have linear quenching by energy transfer, it is necessary that no long lived tryptophan be proximate to more than one NADH binding site.

The difference in the positions of the maxima of the emis-
Fluorescence of Malate Dehydrogenase

Fig. 3. The fitted least squares parameters for fluorescence decay at pH 8.0 as a function of fractional NADH saturation. The different symbols designate data collected on different days. [MDH] = 2 × 10^{-4} M; ionic strength = 0.3. a, decay times; b, weighting factors (scaled to steady state fluorescence).

Fig. 4 (left and center). The fitted least squares parameters for fluorescence decay at pH 6.0 as a function of fractional NADH saturation. [MDH] = 5 × 10^{-3}, ionic strength = 0.3. a, decay times; b, weighting factors (scaled to steady state fluorescence).

Fig. 5 (right). Quenching of steady state LDH emission by I^- and acrylamide at pH 6.0 (○) and pH 8.0 (○).

Table II
Comparison of the fluorescence properties of LDH and s-MDH

<table>
<thead>
<tr>
<th></th>
<th>LDH</th>
<th>s-MDH</th>
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<tbody>
<tr>
<td></td>
<td>(4 subunits, 6 tryptophans/subunit)</td>
<td>(2 subunits, 5 tryptophans/subunit)</td>
</tr>
<tr>
<td>1. Three or more decay components</td>
<td></td>
<td>Probably three or more decay components</td>
</tr>
<tr>
<td>2. A long lived (8 ns) decay component</td>
<td>No 8-ns component</td>
<td></td>
</tr>
<tr>
<td>3. Emission $\lambda_{max} = 337.5$ nm</td>
<td>$\lambda_{max} = 331$ nm</td>
<td></td>
</tr>
<tr>
<td>4. NADH quenching by energy transfer, nonlinear</td>
<td>NADH quenching by energy transfer, linear</td>
<td></td>
</tr>
<tr>
<td>5. Ratio of weighting factors $a_2/a_1 = 1$</td>
<td>$a_2/a_1 = 0.5$</td>
<td></td>
</tr>
<tr>
<td>6. $a_1$ decreases nonlinearly with NADH binding, $a_1$ constant</td>
<td>$a_2$ and $a_1$ independent of NADH binding</td>
<td></td>
</tr>
<tr>
<td>7. $\tau_2$ decreases nonlinearly $\tau_1$ constant</td>
<td>$\tau_2$ decreases linearly with NADH binding, $\tau_1$ constant</td>
<td></td>
</tr>
<tr>
<td>8. Some static acrylamide quenching, more effective than $I^-$</td>
<td>Only dynamic acrylamide quenching, more effective than $I^-$</td>
<td></td>
</tr>
<tr>
<td>9. No pH dependence</td>
<td>No pH dependence</td>
<td></td>
</tr>
<tr>
<td>10. Bound NADH shows two decay components</td>
<td>Two NADH decay components</td>
<td></td>
</tr>
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</table>

a T. Torikata, unpublished results.

The fluorescence spectra is noteworthy. The crystallography of LDH indicates that in the tetramer 16 of the 24 tryptophans are not accessible to solvent (11) and the observed $\lambda_{max}$ is characteristic of proteins with little tryptophan exposure to solvent (12). In the LDH tetramer, Trp-150 and -323 are exposed to solvent. The shorter $\lambda_{max}$ for s-MDH suggests that no tryptophan is appreciably solvent-exposed.

The acrylamide quenching results are intriguing. In the first place, acrylamide is a much better quencher of tryptophan fluorescence for both LDH and s-MDH than is $I^-$. The ability of acrylamide to quench "buried" tryptophan has been well documented (13). The lipophilic interior of the protein appears to concentrate acrylamide. Secondly, acrylamide is a better quencher for s-MDH than LDH. Finally, there is a static contribution to LDH quenching by acrylamide, whereas the s-MDH quenching is dynamic.

For a single emitter, the operational criterion for static quenching is a decrease in $\Phi/T$. The decrease in $\Phi$ is due to formation of a nonfluorescent ground-state complex. When multiple emitters are present, static quenching is signaled by a decrease in $\Phi/\tau$ where $\Phi/\tau = (\Sigma a_i/\Sigma \tau_i)$. This is equivalent to the statement that $\Sigma a_i$ decreases when $\Sigma a_i/\tau_i$ is scaled to the steady state fluorescence intensity. The NADH strong
quenching in LDH is static quenching in this sense. Deviation from steady state Stern-Volmer behavior is not an unambiguous criterion for static quenching (13), but a reduction in $\Sigma a$, can have no other interpretation.

The reduction in $\Sigma a$, for LDH, when compared to the constancy of $\Sigma a$, for s-MDH, suggests that acrylamide binds in close proximity to one or more of the LDH tryptophans and that no such binding occurs to s-MDH.

The preceding discussion has emphasized dissimilarities in the fluorescence properties of LDH and s-MDH. The great sensitivity of tryptophan fluorescence to change in environment is well illustrated by the homologous lysozymes, for which it was found that the decay time of the invariant residue (Trp-108) was very different for the human and hen egg white enzymes (14), which have similar structures. In view of this sensitivity, the similarities between the fluorescence properties of s-MDH and LDH deserve emphasis and can be viewed as significant. Indeed, a great similarity in tryptophan properties becomes apparent when one accepts Trp-248 of LDH, the long lived (8 ns) emitter, as being absent in s-MDH. With this understanding, the five remaining chromophores of LDH, like the five of s-MDH, would be dividable into three of approximately 1-ns and two of approximately 4- to 5-ns lifetime. The value of the ratio $a_2/a_1 = 0.5$, found for s-MDH, is close to the expected value for LDH in the absence of Trp 248. The rationalization of the linear and nonlinear quenching by NADH, observed for s-MDH and LDH, respectively, has been discussed above. The generally similar environments of the tryptophans is reflected in the much stronger quenching by acrylamide than $\Gamma$. Also, the specific binding of phosphate ion to LDH$^\phi$ quenches the long lived component of LDH, and the presumed absence of Trp-248 in s-MDH suffices to explain the absence of an effect of phosphate on s-MDH fluorescence.

To summarize, the differences in fluorescence properties, comparing LDH to s-MDH, may reflect relatively small differences in structure, and indeed the fluorescence behavior of these proteins shows notable similarities. Thus, a measurement very sensitive to local environment supports the homology in overall three-dimensional structure and enzyme chemistry that has been recognized as existing for LDH and s-MDH (1). It is appropriate to point out the similarity of the fluorescence behavior of these proteins becomes apparent through an analysis of the time-resolved measurements, but it was not apparent in a comparison of the steady state fluorescence properties.

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Lifetimes and NADH quenching of tryptophan fluorescence in pig heart cytoplasmic malate dehydrogenase.
T Torikata, L S Forster, R E Johnson and J A Rupley


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