Tryptophan Fluorescence Lifetimes in Lysozyme*

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SUMMARY

Tryptophan fluorescence lifetimes at pH 2 and pH 8 have been obtained for lysozyme and for lysozyme derivatives in which tryptophan-62 or tryptophan-108 or both are nonfluorescent. The lifetimes range from about 0.5 ns to 2.8 ns for the various emitting tryptophans. The tryptophan lifetimes appear to increase with exposure of tryptophan to solvent, but intramolecular contacts, probably with cysteine residues, can considerably shorten the lifetime. Intertryptophanyl interactions can also affect fluorescence lifetimes. The tryptophan-108 lifetime in lysozyme is shorter than in the derivative in which tryptophan-62 is oxidized; this is ascribed to energy transfer from tryptophan-108 to tryptophan-62. From the lifetime results the relative intensities emitted by specific tryptophans can be estimated, and these values also support the existence of intertryptophanyl energy transfer. The emission intensity from tryptophan-62 is greater in the presence of tryptophan-108, and the emission intensity of tryptophan-108 appears to be greater in the absence of tryptophan-62. Conformational effects accompanying chemical modification of tryptophan cannot be completely ruled out, however. The tryptophan-62 lifetime at pH 8 in lysozyme is shorter than in the derivatives, which might indicate a subtle conformational effect. Studies with tri-(N-acetylgalactosamine)-protein complexes indicate that both the tryptophan lifetimes and the number of emitting tryptophans may be changing upon complexation. The results illustrate the usefulness and the limitations of lifetime measurements in understanding protein fluorescence.

The natural fluorescence of proteins is usually dominated by the contribution of the tryptophan residues (1). However, the tryptophan residues in a protein do not necessarily fluoresce equally (2, 3), so that it is useful to isolate the emission of individual tryptophans if possible. Since the emission spectrum of each tryptophan may differ, it is possible, in principle, to resolve the contributions of each by matrix analysis techniques (4). In practice, small differences in the emission spectra of the several tryptophan residues limit the reliability of this procedure. An alternative approach is the use of a transient emission technique, in which the contribution of the tryptophans may be separable in the time domain, i.e. by means of differing excited state lifetimes.

Lysozyme has been used as a model protein because it is well characterized (5), the emission from tyrosine residues is negligible (6), derivatives can be prepared in which 1 or 2 tryptophans are rendered nonfluorescent (5), and the active site tryptophan residues are important to the enzymatic activity of lysozyme (5).

There are 6 tryptophan residues in lysozyme (28, 62, 63, 108, 111, and 123) with Trp-62, Trp-63, and Trp-108 located in the active site. Steady state fluorescence studies have shown that most of the lysozyme emission is due to Trp-62 and Trp-108 (3). The steady state results indicated that the 6 tryptophans are not independent emitters and the existence of nonradiative energy transfer from Trp-108 to Trp-62 was suggested. We have now measured tryptophan fluorescence lifetimes in lysozyme and several derivatives with the hope of resolving the individual contributions to the total emission. Our aim has been to demonstrate the utility and limitations of fluorescence lifetime measurements in proteins with several strong emitters and the consequent use of fluorescence lifetimes as a probe for tryptophan environment.

EXPERIMENTAL PROCEDURES

Hen egg lysozyme was Grade 1 from Miles. The hen lysozyme derivatives and turkey lysozyme were generously provided by Dr. J. A. Rupley. O-62 was prepared by Dr. A. F. Shrage; O-108, E-108, and (NAG)3 were prepared by F. Adams and K. Rudenov; and turkey lysozyme was isolated by Dr. S. K. Banerjee; methods have been described (Refs. 7–11, respectively). The E-108/O-62 protein was prepared by N-bromosuccinimide (NBS) oxidation of ester-108 lysozyme with no attempt at purification. Other chemicals were the best grade available.

Data Collection and Analysis—The instrumentation for the measurement of fluorescence lifetimes has been described (12). The frequency doubled output of a rhodamine 6G dye laser, pumped by an Avco-Everett N2 laser, was used as an excitation source. The data were collected with a Tektronix 661 sampling oscilloscope connected to a Mammotron 400B CAT with analog output digitized and recorded on punched paper tape. Each pulse was represented by 51 points over a range of 33 ns. Increasing the number of points to 200 did not improve the analysis. Averaging 20 to 30 scans of the excitation pulse (monitored by scatter from glycerol solution) or the fluorescence pulse was sufficient. The reliability of the measured lifetimes is indicated by values obtained for 2,5-diphenyloxazole (PPO) in absolute ethanol (1.6 ns) and for anthracene in benzene (4.1 ns) which compare favorably with reported values.

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REFERENCES

1 The abbreviations used are: lysozyme, unless otherwise specified, refers to native hen egg lysozyme, O-62, oxindolealanine-62 lysozyme; O-108, oxindolealanine-108 lysozyme; E-108, oxindoyl (108)-glutamate (35) lysozyme; E-108/O-62, oxindoyl (108)-glutamate (35), oxindolealanine-62 lysozyme; (NAG)3, tri-(N-acetylgalactosamine); GrCl, guanidine hydrochloride.
with previously reported values of 1.6 ns (13) and 4 ns (14), respectively.

Artifacts which can arise in lifetime measurements have been discussed with particular reference to the single photon counting technique (15-17). The following problems are not important in our instrument: (a) photomultiplier misalignment (15): within the limits of our analog data we do not observe multiple peaks in photomultiplier (RCA IP28) response which would indicate this problem; (b) scattered light (16): the Corning 7-51 filter used with our apparatus is known to produce scattered light, and it is estimated that scattering would account for less than 0.1% of the fluorescent signal; (c) wavelength dependence of photomultiplier response (15-17): identical lifetimes were obtained for 2,5-diphenyloxazole emission monitored at 313, 334, and 350 nm. The major limitation in our instrumentation at present is nonrandom noise pickup in the electronics due to the N$_2$ laser discharge. The maximum amplitude of this noise was always less than 0.1%, except for the E-108/O-62 derivative. The concentration of the glycerol solution blank was adjusted to give an absorbance similar to that of the sample. Measurements were at ambient temperature (~23°C) with solutions which were not deoxygenated.

The duration of the excitation pulse, 5 to 7 ns (FWHM), makes it necessary to use a deconvolution technique to analyze the data (14, 18). The decay function, f(t), is related to the observed excitation pulse, P(t), and the observed fluorescence pulse, F(t), by the convolution equation

$$F(t) = \int_{0}^{t} P(t - t')f(t') dt'$$

This equation was solved by assuming a decay function, I = exp(-t/τ), or I = a'exp(-t/τ') + a''exp(-t/τ''), and finding the parameters which on convolution with the excitation pulse gave the best fit to the observed peak of the fluorescence pulse. A nonlinear least squares technique with Gauss-Newton iterations was used. The rms value is used as the criterion of goodness of fit, with

$$\text{rms} = \left(\frac{1}{N} \sum_{i} (F_{obs}^{i} - F_{calc}^{i})^{2} / N_{o} - N_{p}\right)^{1/2}$$

where $F_{obs}^{i}$ and $F_{calc}^{i}$ are the observed and calculated fluorescence intensities in channel i, $N_{o}$ is the number of data channels, and $N_{p}$ is the number of variable parameters in the fitting procedure. Analysis of simulated fluorescence pulses (single or double exponential decay functions convoluted with an experimental excitation pulse) by this technique yielded the input parameters to the third digit.

Knight and Selinger (18) have critically considered several deconvolution techniques and conclude that convolution and fitting is the most satisfactory approach. They also give criteria for deciding on the complexity of the decay function. Grinvald and Steinberg (19) have discussed the least squares technique in detail. The quality of the data collected, i.e., S:N ratio and stray signals determines not only the precision of the computed lifetime but also the number of separate lifetimes that can be resolved. In this work we have used reference compounds whose fluorescence decay is expected to be mono-exponential. 2,5-Diphenyloxazole emission provides a good calibration for lysozyme emission since the 2,5-diphenyloxazole fluorescence lifetime is about the same as the lifetime obtained from the single exponential fit of lysozyme fluorescence; and the emissions are in the same spectral region. No significant decrease in rms value occurred in the analysis of 2,5-diphenyloxazole data in going from a single exponential fit to a double exponential fit; hence a reduction in rms for a double exponential fit is a good indication that the decay is not mono-exponential. We have also used the phase plane plot (20) to help determine if a one exponential decay function is adequate. A linear phase plane plot, such as is found for 2,5-diphenyloxazole data, indicates a single exponential decay, while deviations from linearity indicate a multiple exponential decay, though neither the number of exponentials nor any of the multiple lifetimes can be obtained (20). Phase plane plots of data obtained with our apparatus have been published (21). Thus we have a good reference for a mono-exponential decay, and in addition, the 2,5-diphenyloxazole results show that multiple-exponentiality is not an artifact of our experimental procedures. This, our data permit a distinction between a single and a multiple exponential decay. In addition, a reliable value of the long lived component (if any) can be evaluated in the presence of one or more shorter-lived (<1 ns) contributions. Beyond these considerations, we have confidence in the over-all picture presented below because of the internal consistency which prevails and the general agreement of the data with the lifetime results of other workers (22, 23) and with steady state fluorescence results (3).

## RESULTS

A set of excitation and fluorescence data with the “best fit” convoluted pulse is shown in Fig. 1. The analysis of the data for single and double exponential decays yielded the values shown in the tables, and in all cases a lower rms value is observed for the fit obtained using the double exponential decay function. In the O-62 experiments, the emission of 2,5-diphenyloxazole in absolute ethanol was measured under the same conditions immediately following the O-62 data collection. The 2,5-diphenyloxazole analysis showed no significant decrease in rms in going from a single exponential to a double exponential fit. The phase plane plots (20) gave a straight line for the 2,5-diphenyloxazole data, but not for the O-62 data, thus again indicating multiple exponential decay for O-62, but not for 2,5-diphenyloxazole. We believe that all of our protein data involve contributions from at least two exponentials, but it would not be meaningful to analyze quantitatively for more than two exponentials. The finding of multiple exponential decays is not surprising, since there are 6 tryptophans in lysozyme and it is known from steady state fluorescence results (3) that they do not emit with equal intensity. Chen et al. (22) have reported a single lifetime for lysozyme, and Yashinsky (23) has reported both single and multiple lifetimes for lysozyme and E-108 (which he designated iodine-oxidized-lysozyme). Our lifetime values are only slightly shorter than those in the literature, and our weighting factors ($\phi'$ and $\phi''$) are in good agreement with those of Yashinsky (23).

It is important to note the error limits of the values in Table I.
<table>
<thead>
<tr>
<th>Lysozyme</th>
<th>Single Exponential</th>
<th>Double Exponential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \tau ) RMS</td>
<td>( \alpha_1 ) ( \tau_1 ) ( \alpha_2 ) ( \tau_2 ) RMS</td>
</tr>
<tr>
<td>Lysozyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2(7)</td>
<td>1.90 0.023</td>
<td>0.38 2.67 0.62 0.6 0.0053</td>
</tr>
<tr>
<td>pH 8(22)</td>
<td>1.74 0.017</td>
<td>0.44 2.26 0.56 0.4 0.0076</td>
</tr>
<tr>
<td>pH 2,5 M GuCl (9)</td>
<td>1.96 0.023</td>
<td>0.38 2.69 0.62 0.5 0.0083</td>
</tr>
<tr>
<td>pH 8, 0.1 M KI (9)</td>
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<td>0.50 1.90 0.50 0.2 0.0070</td>
</tr>
<tr>
<td>0-62 Lysozyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2 (7)</td>
<td>1.12 0.016</td>
<td>0.5** 1.52 0.5** 0.3 0.0074</td>
</tr>
<tr>
<td>pH 8 (14)</td>
<td>1.49 0.011</td>
<td>0.43 2.04 0.57 0.7 0.0063</td>
</tr>
<tr>
<td>pH 2,5 M GuCl (11)</td>
<td>1.83 0.023</td>
<td>0.34 2.70 0.66 0.6 0.0080</td>
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<tr>
<td>pH 8, 0.1 M KI (8)</td>
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<td>0.39 2.04 0.61 0.7 0.0075</td>
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<td>E-108 Lysozyme</td>
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<td>1.97 0.023</td>
<td>0.33 2.76 0.67 0.3 0.0057</td>
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<tr>
<td>pH 8 (16)</td>
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<td>1.83 0.015</td>
<td>0.33 2.78 0.67 0.9 0.0057</td>
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<tr>
<td>pH 8, 0.1 M KI (9)</td>
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<td>0.34 2.26 0.66 0.6 0.0063</td>
</tr>
<tr>
<td>E-108/0-62 Lysozyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8.0 (9)</td>
<td>0.70 0.012</td>
<td>0.03 3.0** 0.97 0.5 0.0061</td>
</tr>
<tr>
<td>Lysozyme &amp; Trimer</td>
<td></td>
<td></td>
</tr>
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<td>1.02 0.015</td>
<td>0.15 2.26 0.85 0.6 0.0072</td>
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<td>0.69 2.05 0.31 0.6 0.0060</td>
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<tr>
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<td>1.85 0.012</td>
<td>0.62 2.15 0.38 0.6 0.0081</td>
</tr>
<tr>
<td>E-108 Lysozyme &amp; Trimer</td>
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<td></td>
</tr>
<tr>
<td>pH 2, 2.10^{-4} M (9)</td>
<td>1.49 0.020</td>
<td>0.31 2.42 0.69 0.5 0.0056</td>
</tr>
<tr>
<td>pH 8, 10^{-5} M (9)</td>
<td>1.32 0.017</td>
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<tr>
<td>Turkey lysozyme</td>
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<td></td>
</tr>
<tr>
<td>pH 2 (10)</td>
<td>1.54 0.023</td>
<td>0.24 2.73 0.76 0.6 0.0065</td>
</tr>
<tr>
<td>pH 8 (9)</td>
<td>1.51 0.013</td>
<td>0.38 2.17 0.62 0.7 0.0058</td>
</tr>
<tr>
<td>pH 8, 0.1 M KI (5)</td>
<td>1.30 0.009</td>
<td>0.42 1.75 0.58 0.7 0.0066</td>
</tr>
<tr>
<td>Mean Deviation</td>
<td>5% 10% 15% 5% 15% 25% 10%</td>
<td></td>
</tr>
</tbody>
</table>

* \( a_1 + a_2 = 1.0 \)

† number of determinations included

** error is higher than indicated

The single exponential lifetimes (\( \tau \)) and the double exponential long lifetimes (\( \tau_1 \)) are fairly precise, but the \( \alpha' \) values and particularly the short lifetimes (\( \tau_2 \)) are less so. For this reason we do not consider the variations in \( \tau_2 \) to be significant. However, a larger \( \alpha'_2 \) will be reflected in a shorter \( \tau \) obtained by a single exponential fit, and variations in the single exponential lifetimes as well as in \( \alpha'_1 \) and \( \alpha'_2 \) can be used to diagnose changes in the relative contribution of the long and short lived components. Although the value of \( \tau_2 \) is uncertain, it is unlikely that the existence of this short component is artifactual, especially in view of the large \( \alpha'_2 \) value associated with it.

We draw attention to the following results which will be considered more fully under "Discussion":

1. In E-108/O-62 the small contribution of a long-lived component is probably due to unoxidized E-108; hence the lifetime of emission from E-108/O-62 is about 0.5 ns.
2. For lysozyme, turkey lysozyme, O-108 and E-108 at pH 2, the \( \tau_1 \) are 2.6 to 2.8 ns and are not changed by the addition of 5 M GuCl, which unfolds the protein (24).
3. Whereas \( \tau_1 \) for O-108 and E-108 at pH 8 are identical to those at pH 2, \( \tau_1 \) of hen and turkey lysozyme are shortened to about 2.2 ns at pH 8.
Assignment of Lifetimes in Lysozyme Derivatives—Following Imoto et al. (3), we consider emission from Trp-62, Trp-108, and the composite emission from the R Trps (residues 28, 63, 111, and 123). The results with the E-108/O-62 protein indicate a fast emission (τ ≈ 0.5 ns) from the R Trps. Hence in E-108 and in O-108, τ₁ (Table I) is the lifetime of Trp-62 and τ₂ is the lifetime of the R Trps. In O-62 τ₁ would then be the lifetime of Trp-108 and τ₂ again the lifetime of the R Trps. In the derivatives Trp-62 has a lifetime of 2.6 to 2.8 ns, which is not pH dependent. Trp-108 has a pH-dependent lifetime of 1.5 to 2.0 ns. The R Trps have a very short lifetime of about 0.5 ns.

While it should be kept in mind that Imoto et al. (3) used excitation at 290 nm rather than 280 nm, the lifetimes are generally consistent with the relative fluorescence intensities, F, given by Imoto et al. (3). In the O-62/O-108 derivative, which should be comparable to the E-108/O-62 derivative used in the present work (esterification or oxidation at Trp-108 apparently leads to essentially complete quenching of Trp-108 fluorescence), they found the fluorescence intensity was 15 to 20% that of native lysozyme. The R Trps are highly quenched and consequently a short lifetime would be expected since for similar emitters the lifetimes are often proportional to quantum yields (4). About 50% of lysozyme steady state fluorescence intensity is lost in the O-108 derivative, and somewhat more than 50% lost in the O-62 derivative. The steady state fluorescence intensities are in agreement with a longer lifetime for Trp-62 than for Trp-108. The pH dependence of the steady state fluorescence intensities is somewhat more complicated. F varies with pH in both O-108 and O-62 but in opposite directions. The pH dependence of F in O-62/O-108 is similar to that of F in O-108 and Imoto et al. (3) considered the pH dependence of F in the O-108 protein to arise from the R Trps. The lifetimes also indicate that Trp-62 emission is not pH dependent in O-108, but that Trp-108 emission in O-62 is pH dependent. This pH dependence is probably due to the proximity of Glu-35 (3).

Iodide Quenching—Since τ₁ in O-62 is the lifetime of Trp-108, it is clear that Trp-108 is not quenched by I⁻ as suggested by Lehrer (25). Rather, the O-108 and E-108 results show that Trp-62 is quenched and it appears to be the only tryptophan quenched by I⁻. This is reasonable since Trp-62 and Trp-63 are the tryptophans most exposed to solvent (26) and it is likely that Trp-63 has only a very weak, short-lived emission. The bimolecular quenching constants can be calculated directly,

\[ k_Q = \frac{1}{[I^-]} \left( \frac{1}{\tau_1} - \frac{1}{\tau} \right) \]

where \( \tau_1 \) and \( \tau \) are, respectively, the quenched and unquenched lifetimes. These values are shown in Table II. Combined with the lifetime value this gives a Stern-Volmer constant of about 2 M⁻¹ for lysozyme. This is smaller than the Stern-Volmer constant of 7.5 M⁻¹ obtained by Lehrer (25), but our value does not depend on the assumption of independent and equally absorbing fluorophors which was necessary in Lehrer's work.

Lehrer (25) showed that I⁻ quenching is sensitive to electrostatic charge. In turkey lysozyme, compared to hen lysozyme, a negative charge is removed when Asp-101 (11 A from Trp-62) is replaced by glycine, a change which is consistent with the higher quenching rate. In E-108 a negative charge on Glu-35 (14 A from Trp-62) is removed by esterification at Trp-108, but more precise data would be necessary to determine if the increase in \( k_Q \) is really significant.

Assignment of Lifetimes in Lysozyme—Since there are potentially at least three emitters in lysozyme, we must examine the consequence of analyzing a triple exponential decay for only two exponentials. To approach this question we simulated triple-exponential fluorescence pulses by convoluting a three-component decay function with an experimental excitation pulse. A series of simulations showed that neither of the two lifetimes obtained from the analysis corresponds to any input value unless two of the lifetimes in the triple exponential decay function are similar. In this case the two similar values are averaged to yield one lifetime in the analysis. In particular, the analysis of simulated lysozyme emission, using the Trp-62, Trp-108, and Trp R lifetimes from the derivatives, does not give the observed results at either pH 2 or pH 8. This indicates that the tryptophans of lysozyme are not independent emitters. A satisfactory fit can be obtained at pH 2 by reducing the Trp-108 lifetime to below 1 ns.

The results in 0.1 M KI are of considerable help in the resolution of the lysozyme emission. The similarity of the quenching constants for lysozyme and for O-108, even though the lifetimes are different, indicates that the same emission is being quenched in the two proteins. Since the O-62 results show that Trp-108 is not quenched by I⁻, these data indicate that \( \tau_1 \) of lysozyme at pH 8 is entirely or almost entirely due to Trp-62, and that \( \tau_1 \) and \( \tau_2 \) in lysozyme are sufficiently similar to be averaged together in \( \tau_1 \) of lysozyme. The equivalence of \( \tau_1 \) in lysozyme and \( \tau_0 \) in O-108 and E-108 at pH 2 again suggests that \( \tau_0 \) and \( \tau_2 \) are both included in \( \tau_1 \) of lysozyme.

The lifetime of Trp-108 appears to be shorter in lysozyme than in O-62. But \( \tau_0 \) in lysozyme cannot be too small, since there is evidence indicating substantial direct emission from Trp-108 in lysozyme: (a) the emission spectrum of lysozyme shifts to the red when Trp-108 is oxidized or esterified (3); (b) lysozyme emission shifts to the red with salicylamide energy transfer quenching (27); (c) upon I⁻ collisional quenching, lysozyme emission shifts to the blue (25); and (d) there are significant differences in the fluorescence polarization spectra of lysozyme and E-108 (28).

Although a reliable value cannot be determined for Trp-108 lifetime in lysozyme, a value of about 1 ns would appear to be compatible with the above considerations and with the lifetime data.

In Table III the results of the double exponential fits (Table I)
### Table III

**Lifetime assignments and relative intensities**

<table>
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<tr>
<th></th>
<th>Trp 62</th>
<th>Trp 108</th>
<th>Trp R</th>
<th>Trp 108 + Trp R</th>
<th>(Y^\star)</th>
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<td>Lysozyme</td>
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<td></td>
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<tr>
<td>pH 2</td>
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<td>0.75</td>
<td>0.6</td>
<td>1.00</td>
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<td>0.34</td>
<td>0.77</td>
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<td>1.02</td>
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<td>0.36</td>
<td>0.3</td>
<td>0.24</td>
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<td>0.37</td>
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<td>pH 8</td>
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<tr>
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<td>0.12</td>
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<tr>
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<td>E-108/0-62</td>
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<td>0.6</td>
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<tr>
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<td></td>
<td></td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

\(\delta\) is the relative number of photons emitted by each residue and \(\delta = \alpha_1\). 

**relative to \(\delta\) of lysozyme at pH 2.**

**values in parenthesis may include contributions from other tryptophans.**

are assigned to the specific emitters. The weighting factors \((\alpha'_1\) and \(\alpha'_2\)) in Table I are constrained to sum to 1.0 for each protein. A more meaningful set of weighting factors can be obtained by scaling the transient intensity to the steady state intensity for each protein. The integral of the decay function is an emission intensity measure and

\[
F = \int_0^\infty (\alpha_1 e^{-t/t_1} + \alpha_2 e^{-t/t_2}) dt = \alpha_1 t_1 + \alpha_2 t_2
\]

is a criterion met by \(\alpha_1\) and \(\alpha_2\) in Table III \((F)\) is the steady state intensity with 280 nm excitation relative to lysozyme at pH 5 (3)). In addition the ratio of short component to long component is maintained, i.e.

\[
\alpha_2/\alpha_1 = \alpha'_2/\alpha'_1
\]

In Table III the \(\alpha\) values are all relative to lysozyme at pH 5 and hence comparisons between the different proteins studied becomes possible.

The value \(\alpha_1 t_1\) is then the relative intensity emitted by the \(i\)th fluorophore and is designated as \(\phi\) for each fluorophore in Table III. In most proteins studied here only one emitter contributes to \(\alpha_1 t_1\), and all the shorter lived emitters contribute to \(\alpha_2 t_2\).

Since the \(\alpha_i\) in Table III are properly normalized

\[
\alpha_i = k_i N_0 i
\]

where \(k_i\) is the radiative rate constant for emitter \(i\) and \(N_0 i\) is the relative population of excited states for emitter \(i\) at time zero (assuming a \(\delta\) function excitation pulse). It might appear that all \(N_0 i\) values would be similar since the absorbance of each tryptophan in the protein is nearly the same. But statically quenched residues will behave as if \(N_0 i \approx 0\), and extremely weak emitters will be indistinguishable from background noise and will also exhibit \(N_0 i \approx 0\). The apparent \(N_0 i\) can also be affected by intertryptophanyl energy transfer.

If \(k_i\) is constant for each tryptophan, then for each protein

\[
\sum \alpha = \alpha_1 + \alpha_2 = k_i \sum N_0 i
\]

and \(\sum \alpha\) will give an indication of the number of emitters. In Table III \(\sum \alpha\) for each protein (relative to lysozyme at pH 2) is given. These values are obviously not very precise, but in general they seem most consistent with a total of 3 emitting tryptophans in lysozyme, 2 emitting tryptophans in O-62, O-108, and E-108, and 1 emitting tryptophan in E-108/O-62.

**Turkey Lysozyme**—There are several differences in the amino acid sequence of hen lysozyme and turkey lysozyme; the most important in terms of substrate binding is probably the substitution of glycine in turkey lysozyme for Asp-101 in hen lysozyme (29). The data of Table I show that \(\tau_1\) and \(\tau_2\) are not significantly affected by these changes. However, there does seem to be a greater weighting of the long lifetime component in hen lysozyme,
where at pH 2 it accounts for 38% of the time zero emission compared to 24% in turkey lysozyme. The qualitative reliability of this difference may be seen by a comparison of the single exponential lifetimes, which also indicate a faster fluorescence decay for turkey lysozyme. The single exponential lifetimes will not be sensitive to the absolute intensity of emission but will be dependent on the relative contributions of short and long components and on the actual lifetimes. Thus, the sequence differences between hen and turkey lysozyme do have an effect on the fluorescence properties of the tryptophan residues. The effect seems to be larger at pH 2 than at pH 8, judging from the single exponential lifetimes.

**Tryptophan Environment**—The results indicate that the fluorescence lifetime of tryptophan is sensitive to local environment. Trp-62 is the most exposed to solvent (26) and has the longest lifetime, but exposure to solvent is not the only factor involved. The indole rings of Trp-63, -111, and -123 are more exposed than that of Trp-108 (26) yet the Trp-108 lifetime is much longer according to the O-02 and E-108/O-02 data. An important factor may be intramolecular contacts with sulfur-containing residues. Sulfur-containing compounds are effective collisional quenchers of indole fluorescence (30), and sulfur-containing indole derivatives are only weakly fluorescent (31). Table IV gives side chain contacts of the 10 sulfur-containing residues in lysozyme with the 6 tryptophans. Trp-63, -111, and -123 have large contacts with cystine residues while Trp-28, -108, and -111 have little or no contact with cystines. Trp-28, -108, and -111 have contact with methionine, but according to Cowgill (31) methionine is probably not efficient at quenching tryptophan fluorescence. Contacts with cystine should be particularly important since all cystines in lysozyme are involved in disulfide bonds which will quench fluorescence more strongly than sulfhydryls (31, 32).

Upon unfolding the proteins by guanidine hydrochloride the Trp-108 lifetime becomes equivalent to that of Trp-62, indicating similar environments. However, the short lifetime component ($\tau \approx 0.5$ ns) is still present in the denatured proteins. This is probably due to the remnants of structure maintained by the four disulfide bonds. The quantum yield of denatured lysozyme with the disulfide bonds broken is about 50% greater than that of denatured lysozyme with the disulfide bonds intact (6).

In general, it seems that the fluorescence lifetime of tryptophan increases with exposure to aqueous environment, but specific protein contacts may dominate this solvent exposure dependence.

**Energy Transfer**—Since the Trp-108 lifetime is shorter in lysozyme than in O-62, this means that the presence of Trp-62 increases the rate of excited state Trp-108 decay. This could possibly be due to a conformational effect in the vicinity of Trp-108 caused by the chemical modification of Trp-62 or to energy transfer from Trp 108 to Trp 62 in lysozyme or both.

If energy transfer is occurring, the energy lost by Trp-108 must be gained by Trp-62. This appears to be the case. From Table III it can be seen that, as measured by $\phi$, the number of photons emitted by Trp-62 is greater in lysozyme than in O-108 or E-108. Table III also shows that the combined emission from Trp-108 and Trp R is less in lysozyme than in O-62, but these numbers are less dependable. The steady state results (3) indicate that the energy lost by Trp-108 in lysozyme is salvaged by a stronger emitter at pH 2, since the measured fluorescence intensity of lysozyme is larger than that calculated from the fluorescence intensity of the derivatives.

The lifetime arguments point to substantial energy transfer from Trp-108 to Trp-62 in lysozyme at both pH 2 and pH 8. The steady state results did not indicate energy transfer at pH 8 (3); i.e. the sum of fluorescence intensity of Trp-62, Trp-108, and R Trps, obtained from lysozyme oxindole derivatives, was equivalent to the fluorescence intensity of lysozyme. The lifetime results, however, show that this equivalence may be due to reduced emission from Trp-62 (smaller $\tau_{m}$ at pH 8) rather than to reduced transfer from Trp-108 to Trp-62. Given the experimental uncertainty of the short lifetime component, we can make no statement on the possibility of energy transfer from the R Trps discussed by Imoto et al. (3).

The rate constant for Trp-108 $\rightarrow$ Trp-62 energy transfer is given by

$$k_{tr} = \frac{1}{\tau_{m}(O-62)} - \frac{1}{\tau_{m}(O-62)}$$

This indicates $k_{tr} \approx 10^{8}$ s$^{-1}$ assuming $\tau_{m}$ in lysozyme is 1 ns. With the crystal structure Trp-62, Trp-108 separation and orientations (5), this value appears consistent with a Förster transfer mechanism if the Trp-108 emission, Trp-62 absorption overlap integral is of the order $10^{-17}$ cm$^2$ M$^{-1}$. Although it is difficult to accurately measure this overlap integral, the value is plausible. Eisinger et al. (33) computed overlap integrals of 0.4 $\times$ 10$^{-17}$ for Trp $\rightarrow$ Trp transfer in water, and 2.1 $\times$ 10$^{-17}$ for Trp $\rightarrow$ Trp transfer in chymotrypsinogen.

**Effects of Chemical Modification**—The implicit assumption in using the approach of chemical modification is that oxidation (or esterification) at a particular tryptophan has no effect on the environments of the other tryptophans. The lifetime results raise the possibility that this assumption may not be entirely correct. In Table III it can be seen that at pH 2 the Trp-62 lifetimes are very similar in lysozyme, O-108, and E-108, but the Trp-62 lifetime is shortened in lysozyme at pH 8 and is not affected by the pH increase in O-108 or E-108. Hence there is some effect on the excited state of Trp-62 which apparently depends on the presence of Trp-108. This cannot be due to energy transfer from Trp-108 since this could not change the lifetime of Trp-62 as the energy acceptor.

The origin of Trp-62 lifetime reduction in lysozyme from 2.67 ns at pH 2 to 2.26 ns at pH 8 is not obvious, but it may be an interaction with Trp-63. Whereas Trp-63 does not fluoresce strongly, it may still act as an energy acceptor. Thus quenching of Trp-62 by Trp-63 at pH 8 in lysozyme may be possible, but it is not clear why this does not also occur in O-108 or E-108 unless oxidation or esterification of Trp-108 affects the position of Trp-63. An alternative explanation may lie in the role of solvent.

**Table IV**

**Surface contact of tryptophan with cystine and methionine in lysozyme (see Ref. 20)**

<table>
<thead>
<tr>
<th>Tryptophan Residue No.</th>
<th>Cystine</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residue No.</td>
<td>Contact (Å)</td>
</tr>
<tr>
<td>28</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>62</td>
<td>64</td>
<td>9</td>
</tr>
<tr>
<td>63</td>
<td>76</td>
<td>94</td>
</tr>
<tr>
<td>108</td>
<td>94</td>
<td>36</td>
</tr>
<tr>
<td>111</td>
<td>115</td>
<td>129</td>
</tr>
<tr>
<td>123</td>
<td>30</td>
<td>128</td>
</tr>
</tbody>
</table>

(continues)
complexation in indole emission (34). Tryptophan emits at shorter wavelengths in the fully folded protein than in the denatured protein (3). This is evidence for inhibition of exciplex formation in the folded protein. Because of the geometrical restrictions on solvent structure in the active site cleft (evidenced by displacement of water molecules on binding N-acetylglucosamine (35)), it is conceivable that the effect of modification of Trp-108 is propagated through solvent structure. It should be pointed out that this effect exists only at pH 8; at pH 2 the results are consistent with the assumption of no indirect conformational effects on Trp-62.

The lifetime results do not show any significant differences between the emission of O-108 and E-108 though the quantum yield of E-108 is somewhat lower than that of O-108 (3). We have no explanation for this discrepancy, but it may in some way be due to the unfolding and refolding required for the preparation of O-108 (8).

We emphasize that very subtle conformational effects are being considered here. Imoto et al. (3) have reviewed the evidence which suggests that any conformational changes caused by chemical modification of Trp-62 and Trp-108 would have to be very small. The most convincing points are crystallographic analysis of E-108 (36) and NMR analysis of O-62 (37). Our results with I− quenching indicates that the environment of Trp-62 is very nearly the same in lysozyme, O-108, and E-108.

Saccharide Binding—Tri-(N-acetylglucosamine) will strongly bind lysozyme and E-108 (9), but only weakly bind O-108 (9) or O-62. Since it would be very difficult to correct the lifetime measurements for the effects of incomplete binding, at present we have data on only the lysozyme and E-108 saccharide complexes.

For lysozyme at pH 8 the steady state fluorescence intensity increases on binding (NAG)3, but at pH 2 and 5, and for the lysozyme derivatives at all of the pH values, the steady state fluorescence intensity decreases on binding (3). The lifetime results show that the intensity emitted (φ) by the long lifetime component in lysozyme increases at pH 8 and decreases at pH 2 on binding (NAG)3. However, τ1 is reduced at both pH 2 and 8. At pH 8, the reduction in lifetime is compensated for by an increase in φ, which may mean that emission from another tryptophan is now included with Trp-62 emission in the long lifetime component. At both pH 2 and 8, the possibility exists that a nonemitting tryptophan in lysozyme becomes an emitter in the complex, since the ∑α for the complex is greater than for lysozyme, but the differences may not be significant.

E-108 binding (NAG)3 does not show the same effects as lysozyme. At pH 8, τ1 of E 108 remains the same on binding but α1 decreases. This would normally indicate static quenching, i.e. fewer emitters. However, ∑α is the same at pH 8 for E-108 free or complexed.

The changes in tryptophan fluorescence that occur when lysozyme binds a saccharide cannot be reliably interpreted from the present data. The results do indicate that understanding these fluorescence changes will not be a trivial problem since both the lifetimes and the number of emitting tryptophans may be changing.

CONCLUSIONS

The lifetime results show that Trp-62 makes the largest contribution to lysozyme emission. Teichberg and Sharon (39) implicated Trp-108 as the dominant emitter; this was questioned by Yashinsky (23), and it seems clear from our results that Trp-108 cannot be the major emitter. There may be as few as 3 emitting tryptophans in lysozyme.

The lifetime results meet several criteria expected if nonradiative energy transfer occurs from Trp-108 to Trp-62. The lifetime of Trp 108 is shortened by the presence of Trp 62. The number of photons emitted by Trp-62 is larger when Trp-108 is present, and the number of photons emitted by Trp-108 appears to decrease when Trp-62 is removed. The rate constant for energy transfer is calculated from τ values and appears consistent with the separation, orientations, and spectral properties of Trp-62 and Trp-108. However, whereas this is good evidence for energy transfer, we do not feel that other possibilities, especially subtle conformational changes, can be completely excluded.

The fact that at pH 8 the Trp-62 lifetime in lysozyme is shorter than in O-108 or E-108 indicates that such subtle effects might exist though it is not clear if this particular effect is due to the protein structure or to solvent structure in the active site.

The trypothan lifetimes depend on at least two factors. Increased exposure to solvent appears to increase tryptophan lifetimes, but specific intramolecular contacts, probably with disulfides, may make a tryptophan a nonemitter or a very short-lived emitter.

The quenching results with I− indicate that a charged quencher can be very sensitive to the location of charges on the protein molecule. Asp-101 has an effect and Glu-35 may have an effect on the quenching of Trp-62 though the charged groups are, respectively, 11 and 14 Å away. The size of the quencher can be very important also. I− only appears to quench Trp-62, but O2 appears to quench even buried tryptophans (39, 40).

The importance of lifetime measurements in conjunction with quantum yield measurements in protein fluorescence work is demonstrated by these results: the fluorescence contributions of individual tryptophans were resolved; relative emission intensities of specific tryptophans were obtained; a much clearer picture of possible intertrypothan energy transfer was achieved; fluorescence lifetimes were found to depend on trypothan environment, and finally, the difficulties of extension of information on the fluorescence of free lysozyme to the fluorescence of the saccharide complex of lysozyme were seen.

Perhaps the greatest limitation in using lifetime measurements to analyze protein emission is the inability to analyze quantitatively for more than two or perhaps three exponential contributions. A closely related problem is the limitation of resolving lifetimes which are only slightly different. Single photon counting techniques inherently have a better time resolution than our detection system, but even with the lower noise data of single photon counting, analysis for a multiple exponential decay is difficult (18). There appears to be no easy way to solve these problems in the near future, but within these limitations lifetime measurements are capable of yielding much information on protein fluorescence.

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