Subpicosecond Fluorescence Depolarization Studies of Tryptophan and Tryptophanyl Residues of Proteins

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Fluorescence depolarization of the exposed tryptophanyl residue in the peptide melittin and the buried tryptophanyl residue in the protein azurin (from the bacteria Pseudomonas aeruginosa) was measured with subpicosecond resolution at various excitation and emission wavelengths. A short time component was found in both the fluorescence decay and fluorescence anisotropy decay for the tryptophanyl residue in melittin with time constants of 1.4 ± 0.2 and 3.8 ± 0.2 ps, respectively. The short time component is attributed to both singlet excited states being nearly degenerate and the rate of internal conversion between the two lowest lying singlet excited states. The limiting value of the initial anisotropy of 0.40 ± 0.02 is observed for the tryptophanyl residue in melittin when excited with 300-nm light. These results are similar to those observed for tryptophan in water. The fluorescence depolarization measured for the tryptophanyl residue in azurin did not exhibit a short time component, which is attributed to both singlet excited states being nearly degenerate and the rate of internal conversion beyond our temporal resolution. We propose a new model for the fluorescence depolarization observed in the first few picoseconds for tryptophan in water and for tryptophanyl residues in proteins, which takes into account conformational heterogeneity and the effect this has on population exchange between the two low-lying singlet excited states.

Introduction

Fluorescence from tryptophanyl residues in proteins has been widely used to investigate both structure and dynamics. As a probe, the tryptophanyl residue has several advantages; of the three naturally occurring aromatic amino acids it has a significantly higher fluorescence quantum yield and radiative rate, and its fluorescence spectrum is strongly influenced by its environment. Consequently, there is great interest in its photophysics. Absorption and fluorescence of tryptophan occur in the near-ultraviolet part of the spectrum, and both have their contributions from two low-lying singlet excited states in the indole moiety designated 1L<sub>u</sub> and 1L<sub>g</sub>.<sup>10-13</sup> Fluorescence originating from the 1L<sub>u</sub> state in indoles is greatly red-shifted in the presence of polar solvents, whereas fluorescence originating from the 1L<sub>g</sub> state is comparatively insensitive to the environment of the indole ring. Molecular dynamics (MD) simulations predict that thermal fluctuations occurring on a picosecond time scale are relevant to large-scale motion in proteins.<sup>15-17</sup> Northrop et al.<sup>18</sup> and Cartling<sup>19</sup> have utilized theories of stochastic processes to connect the short time fluctuations obtained from molecular dynamics simulations with the long time conformational changes important for protein function. The bridge between the two time regimes is the evaluation of the rate of conformational transitions. While fluorescence anisotropy measurements have been an important experimental tool for studying these motions, they provide only an important experimental source of long time dynamic information on proteins and peptides. Experimental evaluation of short time fluctuations is lacking. Fluorescence depolarization measurements on a few picosecond time scale may reveal thermal fluctuations within conformational states and provide a benchmark for further MD simulations. The lack of these measurements mean that large and uncertain extrapolations are necessary in efforts to analyze the effectiveness of MD simulations in predicting protein motions. Recently, Ruggiero et al.<sup>13</sup> provided strong evidence that population exchange between the 1L<sub>u</sub> and 1L<sub>g</sub> states for tryptophan in water adds a nonrotational contribution to the fluorescence anisotropy within the first 5 ps after excitation.<sup>13</sup> A model describing the rapid fluorescence depolarization which takes into account excitation of both the 1L<sub>u</sub> and 1L<sub>g</sub> states followed by rapid internal conversion between these two states was first suggested by Cross et al.<sup>22</sup> The rate of internal conversion is related to the energy separation between the two states by the energy gap law and reflected in the fast components of both the population and anisotropy fluorescence decays. This model was extended by Ruggiero et al.<sup>13</sup> to include vibronic relaxation, which gives rise to an excitation wavelength dependence of the initial fluorescence anisotropy. Consistent with this model for fluorescence depolarization of tryptophan, Ruggiero et al. fit their fluorescence anisotropy data globally over an excitation and emission surface assuming that both the population and fluorescence anisotropy decays had no excitation or emission dependence. They reported a large-amplitude rapid decay component in both the population and fluorescence anisotropy decays at emission wavelengths of 330 and 335 nm for 300-nm excitation of tryptophan, while at redder emission wavelengths anisotropy components with negative amplitudes were reported.

(6) Cantor, C. R.; Schimmel, P. R. In Biophysical Chemistry; Freeman: San Francisco, 1980; Part II.

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Tryptophan and Tryptophanyl Residues of Proteins

The purpose of the present paper is to determine whether population exchange between the \( L_1 \) and \( L_2 \) states occurs in tryptophanyl residues of proteins and peptides and to what extent this limits the ability to extract information about the short time dynamics from fluorescence depolarization measurements. To this end we have studied tryptophanyl residues in two different protein environments. In melittin, a 26 amino acid peptide with a random coil structure, the tryptophanyl residue is exposed to the solvent and its fluorescence is red-shifted.\(^{25}\) The protein azurin from the bacterium \textit{Pseudomonas aeruginosa} (azurin Pae) has a tryptophanyl residue buried in the protein away from the solvent\(^{26-28}\) and has a fluorescence maximum at 308 nm\(^{29}\)—the bluest known for any protein.

In the analysis presented by Ruggiero et al.,\(^{13}\) the existence of conformers of tryptophan and their influence on the population and anisotropy decays were not considered. In the more detailed analysis presented in this work, we find it necessary for both tryptophan and melittin to extend the model discussed above to include conformational heterogeneity.\(^{30-36}\) The contributions from species with different emission spectra\(^{30}\) make the global linkages used in the earlier fitting procedure inappropriate. For emission wavelengths less than 340 nm the two analyses give very similar results. However, for emission wavelengths \( \geq 340 \text{ nm} \) significant differences are observed. In particular, the negative amplitudes in the anisotropy decays presented by Ruggiero et al. seem to be an artifact of the global linkages assumed in their homogeneous model.\(^{37}\)

**Experimental Section**

The ultraviolet fluorescence upconversion system used in our experiments is described in detail elsewhere.\(^{31}\) Briefly, subpicosecond pulses were generated by a hybrid dye laser with intracavity chirp compensation synchronously pumped by the second harmonic of a mode-locked Quantronix Nd:YAG laser. Auto-correlations of dye laser pulses centered at 584 and 600 nm had a full width at half-maximum (fwhm) of 1 ps and 700 fs, respectively. These pulses were amplified by a five-pass bow tie amplifier pumped at 6 kHz by an Oxford Cu 40 copper vapor laser. The second harmonic of the amplified pulse was used to excite the sample, and fluorescence was collected in 180° geometry. The structured absorption around 360 nm indicative of the monomer (the tetramer has an emission maximum around 330 nm).\(^{20}\) The structured absorption spectrum of tryptophan collected at emission \( \lambda = 308 \text{ nm} \) was kept at 1°C while flowed in the absence of air through a 1 mm silicated quartz cuvette and silicone tubing using a Cole Palmer peristaltic pump at a rate of 3 cm/s. Melittin samples were monitored for deterioration before and after an experiment by fluorescence and always had an emission maximum around 360 nm indicative of the monomer (the tetramer has an emission maximum around 330 nm).\(^{20}\) The structured absorption and very blue fluorescence of azurin Pae were not detectably altered throughout an experiment. Melittin did show small amounts of aggregation as determined by the appearance of scatter in the absorption spectrum. However, its fluorescence spectrum remained unaltered and there was no appreciable sign of tetramer formation. A melittin sample was used for only two experiments, and the typical duration of an experiment was 3–4 h.

**Results**

The fluorescence anisotropy is related to the fluorescence intensities that are parallel, \( I_{//}(t) \), and perpendicular, \( I_{\perp}(t) \), to the excitation polarization through the equations:\(^{15,40}\)

\[
\tau(t) = \frac{I_{//}(t) - I_{\perp}(t)}{I_{\perp}(t) + 2I_{//}(t)} 
\]

**Figure 1.** Parallel fluorescence decay components of the anisotropies from 300-nm excitation of melittin collected at emission wavelengths of 325, 330, 335, 340, and 360 nm, and of tryptophan collected at emission wavelengths of 330, 335, 340, and 360 nm.
Global analysis can be misleading when the underlying physical behavior is more complex than assumed in the global model.

For global analysis the time constant for each component in both parallel and perpendicular decays along with the anisotropy decay from the 330-nm emission of melittin excited at 300 nm. Noteworthy is the unique shape of the perpendicular decay, in particular, the sharp rise, which is characteristic of the influence of level kinetics in the anisotropy. The anisotropy decay has an initial value of 0.40 ± 0.02. To the best of our knowledge, such a high initial fluorescence anisotropy has not been previously observed for a tryptophanyl residue in a protein. We have measured, within an experimental error of 0.02, an initial anisotropy of 0.40 for all emission wavelengths from excitation of melittin with 300-nm excitation (see Table 1).

A comparison of the parallel and perpendicular decays from 292-nm excitation of melittin and azurin is presented in Figure 2. Two important differences in the 292-nm melittin data as compared at 300 nm are a decrease in the amplitude of the fast component in the parallel decay and a much lower initial anisotropy (see Figure 4). However, one still observes a sharp rise in the perpendicular decay curve. Since the absorbptivity at 300 nm for azurin is very small and its fluorescence very blue-shifted, we measured decays for 292-nm excitation and 314-nm excitation (see Table 4). However, important differences in the 292-nm melittin data as compared at 300 nm are a decrease in the amplitude of the fast component in the parallel decay and a much lower initial anisotropy (see Figure 4). However, one still observes a sharp rise in the perpendicular decay curve. Since the absorbptivity at 300 nm for azurin is very small and its fluorescence very blue-shifted, we measured decays for 292-nm excitation and 314-nm excitation. There is no apparent fast component in either the parallel or anisotropy decays for azurin (see Figure 3); however, the perpendicular decay does have a fast rising edge. Apoazurin also exhibits a low initial anisotropy (r(0) = 0.20 ± 0.02) for 292-nm excitation.

Data Analysis

The melittin data sets (composed of the parallel and perpendicular decay curves) measured at the various excitation and emission wavelengths were fit individually and by global analysis. The results are summarized in Table 1. We assume a two-exponential fluorescence decay, \( K(t) \), and anisotropy decay, \( r(t) \), for each data set:

\[
K(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) \quad (5)
\]

\[
r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) \quad (6)
\]

For global analysis the time constant for each component in both the fluorescence and anisotropy decays was globally linked by the data sets. Implicit in the global linkage is the assumption that the time constants have no intrinsic wavelength dependence. Global analysis can be misleading when the underlying physical behavior is more complex than assumed in the global model. A reanalysis of the trypophan data combined with the new melittin data leads us to the conclusion that the differences between the fluorescence spectra of the tryptophan conformers cannot be ignored, and therefore the time constants for the different data sets cannot be globally linked. To illustrate this point, we will present the results of the global analysis and simulate the data with a model which does not include spectral inhomogeneity before discussing a model which incorporates the spectral differences of the tryptophan conformers.

Since our data were collected over 20-ps time delays, longer time components are poorly determined in a free fit. Consequently, the time constants for the longer components in our global fits were fixed to values obtained from fluorescence and anisotropy decay measurements made on melittin by Lakowicz et al. 24

Though these measurements do not have the temporal resolution to detect the few picosecond component in the fluorescence and anisotropy decays, the longer time components were well characterized. We set the longer decay constant, \( \tau_2 \), to 320 ps and the longer anisotropy time constant, \( \phi_2 \), to 170 ps. The quality of the global fit and the resulting values obtained for the short time components are quite insensitive to the time constants used for the long time components, and a change in their values by ±50% does not alter the fit, within experimental error.

Global analysis over the excitation and emission surface for the tryptophan residue in melittin yielded constants for the fast components in the fluorescence and anisotropy decay of 1.4 ± 0.2 ps and 3.9 ± 0.2 ps, respectively.
and 3.8 ± 2 ps, respectively, with a global $\chi^2$ value of 1.5. The single data set fits had $\chi^2$ values less than 1.2. The time constants determined for the fast component in the fluorescence decays from single data set fits were within 0.5 ps of the value obtained from global analysis. However, the time constants of the fast anisotropy component obtained from single data set fits varied from 2 to 15 ps, with closer agreement to the globally determined value for those data sets obtained from emission wavelengths less than 340 nm.

**Simulations**

A model describing the fluorescence depolarization of tryptophan was first proposed by Cross et al.\(^\text{(1)}\) This model assumes that the $^1$La and $^1$Lb states are simultaneously excited, and emission from both is detected with a relative efficiency which depends on the detection wavelength. In this model the rates of internal conversion between the two states are linked by microscopic reversibility and therefore depend on their energy separation. The absorption and emission transition dipole moments of each state are assumed to be parallel to each other. The angle between the transition dipole moments of the $^1$Lb and $^1$La state is a parameter in the model. We will refer to this model as the single angle model. Ruggiero et al.\(^\text{(13)}\) incorporated vibrational relaxation into the model to explain the excitation wavelength dependence observed for the component obtained from single data set fits varied from 2 to 15 ps, with closer agreement to the globally determined value for those data sets obtained from emission wavelengths less than 340 nm.

### Table I: Global Analysis Results for the Anisotropies of Melittin in Water at 10 °C

<table>
<thead>
<tr>
<th>$\lambda_{ex}$, ±5 nm</th>
<th>r(0)</th>
<th>$\tau_1$</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>0.40 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>330</td>
<td>0.39 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>335</td>
<td>0.38 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>340</td>
<td>0.41 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>360</td>
<td>0.38 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>-0.02 ± 0.02</td>
</tr>
<tr>
<td>292</td>
<td>0.32 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>335</td>
<td>0.11 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>350</td>
<td>0.23 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>-0.01 ± 0.02</td>
</tr>
</tbody>
</table>

* $r(t) = r_1 \exp(-t/\tau_1) + r_2 \exp(-t/\tau_2)$, $\phi_1 = 3.8 \pm 2$ ps, $\phi_2 = 170$ ps; $K(i) = \alpha_1 \exp(-r/\tau_1) + \alpha_2 \exp(-r/\tau_2)$, $\tau_1 = 1.4 \pm 0.2$ ps, $\tau_2 = 320$ ps, $\alpha_1 + \alpha_2 = 1$.

(4) The absorption and emission cross sections as a function of wavelength. Decomposition of the tryptophan absorption spectrum into its $^1$Lb and $^1$La constituents at 77 K in a propylene glycol glass has been reported by several groups.\(^\text{(10,47)}\) Use of these data directly does not seem appropriate since significant shifts may occur on going to room-temperature aqueous solution. Comparison of the room- and low-temperature spectra shows a 3- to 6-fold increase in total absorption at 300 nm and a discernible decrease in absorptivity at 292 nm as a result of significant broadening of the $^1$Lb 0–0 band.\(^\text{(48,49)}\) There is also some broadening of the $^1$La band. Thus, there may be significantly higher $^1$Lb absorption at 300 nm than would be predicted from the low-temperature spectral decomposition. The values of the excitation ratio used in our simulations were 0.35 and 0.20 for 300- and 292-nm excitation, respectively.

The emission detection efficiencies (percentage of total emission cross section due to $^1$Lb) are also difficult to determine experimentally. Estimates were obtained from the steady-state emission spectra of azurin Afe and Pae samples with the same absorbance (0.13) at 280 nm. Azurin Pae is believed to be an $^1$Lb emitter,\(^\text{(50)}\) and azurin Afe an $^1$Lb emitter.\(^\text{(50,51)}\) The detection ratios determined from the azurin spectra (error ±0.05) are 0.77, 0.74, 0.65, 0.55, 0.42, and 0.37 at 325, 330, 335, 340, 350, and 360 nm, respectively.

For wavelengths between 325 and 340 nm these values are similar to those used in the melittin simulations and are within 10% of the values used in the tryptophan simulations; but at longer wavelengths the values are too small to adequately simulate either the melittin or tryptophan data.

(5) The angles between the absorption and emission transition moment directions, $\Theta^{ab}$, $\Theta^{ba}$, $\Theta^{0b}$, and $\Theta^{0a}$. The angles necessary to simulate the data from 300-nm excitation of both tryptophan and melittin are $\Theta^{ab} = 0 = \Theta^{ba}$ and $\Theta^{0b} = 0 = \Theta^{0a}$. We were able to simulate the raw experimental data for both tryptophan and melittin using the four-angle model. Several points emerge from these simulations. First, the only significant difference between the simulations for melittin and those for tryptophan appears in the emission detection ratios. Second, the single-angle model is capable of simulating the data from 300-nm excitation of both tryptophan and melittin; however, the four-angle model is necessary to simulate data with 292-nm excitation, because of the low initial anisotropy. A point of concern is that a rather small angle (25–35°) between the $^1$Lb and $^1$Lm emission dipole moments is required to simulate the data. This is in disagreement with other data that suggest this angle should be near 90°.\(^\text{(52,53)}\) This result led us to reanalyze the tryptophan data of

component A decreases with increasing emission wavelength and determined by Rayner and Szabo. The relative weight of component A and B (i.e., the relative initial intensities of their simulated magic angle decay) is equal to the ratio of the preexponential weights for each component multiplied by the ratio of the preexponential terms of the long decay component relative to the short. The relative weights for the two components as determined by Szabo and Rayner, we are able to adequately simulate the tryptophan data while keeping the angle between the emission dipole moments required by the four-angle model to simulate the tryptophan data for 300-nm excitation creates serious doubts about the model. However, the four-angle model, as it stands, is not a complete description of tryptophan fluorescence. On a nanosecond time scale, the fluorescence decay of tryptophan has two components. Szabo and Rayner have decomposed the emission spectrum of tryptophan into a 500-ps and a 3.1-ns component. Similar results were obtained by Petrich et al. The relative contribution of the two components to the total emission spectrum varies with emission wavelength—the 500-ps component has an emission maximum at 335 nm, while the 3.1-ns component peaks at 355 nm. These two components are assigned to two separate species and have been discussed extensively in terms of the conformer model of tryptophan. By enlarging the four-angle model to include sample heterogeneity (i.e., a distribution of conformers with different absorption and fluorescence spectra) and using the relative weights for the two components as determined by Szabo and Rayner, we are able to adequately simulate the tryptophan data while keeping the angle between the 1L_s and 1L_a emission transition moments of each component at 90°. Although these relative weights were determined from 280-nm excitation of tryptophan, Table I of ref 31 shows that the relative weights of the two components do not vary greatly with excitation wavelength in the range 280–305 nm. This new model assumes that the relative contributions, at a particular wavelength, from the 1L_s and 1L_a states for absorption and emission are in general different for the various conformers of tryptophan.

Table II lists the parameters used in this model to simulate the data from 300-nm excitation of tryptophan. As before, the model reduces to the single-angle model for each component, but now with Φ = 90°. The excitation ratios used to simulate data for 300-nm excitation of the 500-ps component (component A) and the 3.1-ns component (component B) were 0.50 and 0.10, respectively. The emission detection ratio for both components peaks near 335 nm. In this model, the relative weights of components A and B (i.e., the relative initial intensities of their simulated magic angle decays) control the fraction of fluorescence originating from each component. The relative weights of components A and B are adjusted in the simulation such that, at each wavelength, the ratio of the preexponential terms of the long decay component in the magic angle decay for each component multiplied by the relative weight is equal to the ratio of the preexponential weights determined by Rayner and Szabo. The relative weight of component A decreases with increasing emission wavelength and becomes negligible at 380 nm. All other parameters for components A and B were assigned the values used above in the four-angle model to simulate the tryptophan anisotropies.

In Figure 5 parallel and perpendicular fluorescence decays simulated from this model are compared to those measured at 335 nm of tryptophan excited at 300 nm. Figure 6 displays the simulated parallel and perpendicular fluorescence decays for the individual components A and B, which when added together by the appropriate weighting factors yield the anisotropy components displayed in Figure 5. Our simulations clearly show that the short component in both the magic angle and parallel fluorescence

### Table II: Parameters Used in Fluorescence Depolarization Simulations of Tryptophan

<table>
<thead>
<tr>
<th>λ_ex, nm</th>
<th>λ_em, nm</th>
<th>ratio of component A</th>
<th>excitation ratio, % (1L_s/(1L_s + 1L_a))</th>
<th>emission detection efficiency, % (1L_s/(1L_s + 1L_a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>330</td>
<td>0.41</td>
<td>0.50</td>
<td>0.10</td>
</tr>
<tr>
<td>335</td>
<td>330</td>
<td>0.38</td>
<td>0.50</td>
<td>0.10</td>
</tr>
<tr>
<td>340</td>
<td>331</td>
<td>0.24</td>
<td>0.50</td>
<td>0.10</td>
</tr>
<tr>
<td>380</td>
<td>331</td>
<td>0.00</td>
<td>0.50</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The following parameters are used for simulating both components A and B: \(k_{A} = 0.625 \text{ ps}^{-1}\); \(k_{B} = 0.044 \text{ ps}^{-1}\); \(k_{A} = k_{B} = 2.87 \times 10^{3}\); \(1/6\delta_{nm} = 45 \text{ ps}; \Phi = 90^\circ\). Components A and B are the 500-ps and 3.1-ns components, respectively, in the magic angle decay. The ratio of component A is determined from the relative intensity of the simulated magic angle decay of component A at time = 0 to that of component B.

**A Multiconformational Model**

Ruggiero et al. in terms of a model which considers spectral inhomogeneity arising from multiple tryptophan conformers.

The small angle between the emission dipole moments required by the four-angle model to simulate the tryptophan data for 300-nm excitation creates serious doubts about the model. However, the four-angle model, as it stands, is not a complete description of tryptophan fluorescence. On a nanosecond time scale, the fluorescence decay of tryptophan has two components. Szabo and Rayner have decomposed the emission spectrum of tryptophan into a 500-ps and a 3.1-ns component. Similar results were obtained by Petrich et al. The relative contribution of the two components to the total emission spectrum varies with emission wavelength—the 500-ps component has an emission maximum at 335 nm, while the 3.1-ns component peaks at 355 nm. These two components are assigned to two separate species and have been discussed extensively in terms of the conformer model of tryptophan. By enlarging the four-angle model to include sample heterogeneity (i.e., a distribution of conformers with different absorption and fluorescence spectra) and using the relative weights for the two components as determined by Szabo and Rayner, we are able to adequately simulate the tryptophan data while keeping the angle between the 1L_s and 1L_a emission transition moments of each component at 90°. Although these relative weights were determined from 280-nm excitation of tryptophan, Table I of ref 31 shows that the relative weights of the two components do not vary greatly with excitation wavelength in the range 280–305 nm. This new model assumes that the relative contributions, at a particular wavelength, from the 1L_s and 1L_a states for absorption and emission are in general different for the various conformers of tryptophan.

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directly obtain the ... group. Since the steady-state fluorescence spectrum has a maximum near 330 nm. This
spectra of other indoles. The fluorescence spectrum determined by comparing the spectra of the components with the emission
decays and (b) enables the extraction of dynamical information from the anisotropy measurement free of distortion or obfuscation
by way of the four-angle model. There is no independent means to determine these four angles, and with the increased complexity of conformational heterogeneity we do not consider it useful to simulate these data.

Comparing the parallel decays from the tryptophanyl residue in melittin to those from tryptophan in water (Figure 1), one observes very similar trends in the excitation and emission dependence. The values determined for τ, and φ, for the tryptophanyl residue in melittin are the same within experimental error as those determined for tryptophan in water, suggesting that both indole moieties have very similar energy gaps between the two singlet excited states.

It is apparent that simulation of the fluorescence depolarization of the tryptophanyl residue in melittin requires the inclusion of conformational heterogeneity in the model. At least three components appear in the long time fluorescence decay of melittin, suggesting that a greater degree of conformational heterogeneity may exist for the tryptophanyl residue in melittin than for tryptophan in water. This increased complexity makes simulating the fluorescence depolarization of the tryptophanyl residue in melittin impractical at present.

Discussion

The form of the anisotropy decays, in particular the characteristic shape of the perpendicularly polarized emission curve with its sharp rising edge, strongly suggests that level kinetics play a major role in the anisotropy decay of the tryptophanyl residue in melittin. This is particularly clear for the data collected with 300-nm excitation (Figure 2).

The major goal of this work is to arrive at a model for tryptophan and tryptophanyl photophysics that (a) self-consistently rationalizes the complex picosecond population and anisotropy decay and (b) enables the extraction of dynamical information from the anisotropy measurement free of distortion or obliteration by level kinetics.

The multiconformational model we propose has two layers of complexity: the two spectral components arising from conformational heterogeneity each have differing contributions from the 1Ls and 1Lg states. In order to successfully apply this model, we need to know the spectra of components A and B, as well as the 1Ls and 1Lg spectra for each component. A qualitative description of the 1Ls and 1Lg spectrum for each component can be obtained by comparing the spectra of the components with the emission spectra of other indoles. The fluorescence spectrum determined for component A is similar to that of the indole derivative yohimbine, which has an amine that is conformationally constrained.

In yohimbine the 1Ls state is lower in energy than the 1Lg state. When the amine is protonated, the 1Ls state is blue-shifted and the fluorescence spectrum has a maximum near 330 nm. This blue shift parallels the appearance of component A in the tryptophan emission that occurs when the pH is below the pK value of the ammonium group. Since the steady-state fluorescence of yohimbine originates primarily from the 1Ls state, we cannot directly obtain the 1Lg emission spectrum. However, 5-methoxyindole emits primarily from the 1Lg state in water with a maximum at 330 nm and should provide a reasonable model of the 1Ls emission for other indoles in water, since the energy of the 1Ls state in indoles is not greatly perturbed by the local environment or ring substituents.

It is interesting that the position of the emission maxima of component A, yohimbine, and 5-methoxyindole closely corresponds to the wavelength where the amplitudes of the fast components in the anisotropy and magic angle decays are maximum. Also, the position of the emission maximum of 5-methoxyindole corresponds to the maximum value of the emission detection ratios used for components A and B in simulating the fluorescence depolarization of tryptophan (see Table II). Since the fluorescence lifetimes of components A and B are significantly longer than the time scale of internal conversion from the 1Ls to 1Lg state, the zero-time emission spectra for the 1Lg states of each component should be nearly identical to the steady-state spectra determined for each component.

We expect the time-zero emission spectra of the 1Lg states of each component to be similar to that of 5-methoxyindole in water. From the parameters used in our model, we have constructed a crude representation of the zero-time emission spectra for components A and B (left panel, Figure 8), for the 1Ls and 1Lg constituents of component A (center panel), and for the 1Ls and 1Lg constituents of component B (right panel).

The data presented were determined from our simulations of the fluorescence depolarization of tryptophan using the multiconformational model. From these points we are able to determine the position of the maxima within a few nanometers of the time-zero emission spectra of component A and the 1Ls state of both components A and B. However, these few points are insufficient to determine the position of the maxima of either component B or the 1Ls state of the two components. To fill in this missing information we relied on the resolution of the steady-state fluorescence spectra of tryptophan into two components by Szabo and Rayner. As previously mentioned, component B in our model is related to the component determined from the resolution of the steady-state fluorescence spectrum that has a maximum at 355 nm. On the basis of this information, we estimate the shape of time-zero emission spectra of component B and the 1Ls state of both components between 350 and 380 nm. These initial emission spectra provide a qualitative picture of the model being proposed and may provide a guide for the design of future studies.

We conclude from our model that the large amplitude of the fast component in the magic angle and anisotropy decays originates primarily from component A due to the high excitation and detection ratios. Although component B has a high detection ratio at the blue edge of the emission, the excitation ratio is small, leading to significantly smaller amplitude of the fast component.

The similarities observed between the parallel and perpendicular fluorescence decays of tryptophan and the tryptophanyl residue
in melittin do not carry over to those measured for the buried tryptophanyl residue in azurin Pae. The emission from tryptophan 48 of azurin Pae has generally been ascribed to $1_{L_b}$ fluorescence, and clearly the $1_{L_a}$ state is substantially blue-shifted compared to the tryptophanyl residue in melittin. A similar blue shift of the $1_{L_a}$ state has also been observed for N-stearyl-l-tryptophan n-hexyl ester dissolved in methycyclohexane. The measured anisotropy for azurin Pae has a low $r(0)$, about 0.20, with no detectable fast component in either the anisotropy or fluorescence decays. The simplest explanation consistent with this finding is one in which the $1_{L_a}$ and $1_{L_b}$ states are nearly degenerate, and the rate of internal conversion is faster than the temporal resolution of our instrument. Both population and anisotropy decays fit well to a single-exponential function; thus, no rapid motion of the buried tryptophan is detected, consistent with molecular dynamics simulation results.

Finally, we turn to the question of whether rapid internal motion is detected in our melittin measurements. It is clear from the foregoing discussion that, at least for emission wavelengths <350 nm, the fluorescence depolarization of melittin is dominated at short times by the $1_{L_a}/1_{L_b}$ level kinetics. However, given the low $L_a$ excitation ratio at 300 nm and the diminished $1_{L_b}$ emission at wavelengths longer than 350 nm, we conclude that for exposed tryptophanyl residues in proteins excitation at 300 nm and detection at 380 nm removes the contribution of level kinetics from the anisotropy. Of course, complications resulting from $1_{L_a}$ emission from a distribution of conformers will remain, and experimental verification of this proposal is clearly necessary.

Recently, Axelsen et al. compared molecular dynamics simulations of protein-based and time-resolved fluorescence anisotropy measurements. However, their comparison is based on the orientation of the emission dipole moment being $\pm 25\degree$ to the absorption dipole moment. They determined this value from the low initial fluorescence anisotropy of 0.29 when tryptophan is excited with 295-nm light. They arrived at this angle for essentially the same reason we needed to use an angle of around 30\degree to simulate the fluorescence depolarization of tryptophan and melittin using the single-angle model. As we have pointed out, this artificially small angle is the result of level kinetics which are further complicated by conformational heterogeneity.

Concluding Remarks

The fluorescence anisotropies of buried and exposed tryptophanyl residues show markedly different fluorescence anisotropy decays. The exposed tryptophanyl residue in melittin is very similar to tryptophan in its fluorescence behavior. The previous model by Ruggiero et al. to describe the fluorescence depolarization of tryptophan has been shown to require revision. Tryptophan can be successfully described by considering internal conversion between two overlapping states that are perturbed to varying degrees by the different environments of the indole moiety resulting from conformational heterogeneity. An analogous model for tryptophanyl residues in proteins is evident. The rate of $1_{L_b}$ to $1_{L_a}$ internal conversion in melittin is determined to be $1.4 \pm 0.2$ ps at the blue edge of the emission, which is within error identical with the rate in tryptophan ($1.6 \pm 0.2$ ps). The internal conversion contributes a nonrotational contribution to the anisotropy decay of $3.8 \pm 2$ ps. Our measurements are, we believe, the first observation of an initial fluorescence anisotropy of 0.40 for a tryptophanyl residue in a protein.

The shift in energy levels which accompanies a change in tryptophan environment from polar to nonpolar changes the fluorescence behavior markedly. No rapid component could be detected in the fluorescence anisotropy of the buried W48 in azurin Pae; however the initial anisotropy is low, and we suggest that the interlevel relaxation is complete on a time scale shorter than our resolution ($\leq 500$ fs).

Finally, we suggest that excitation of a solvent-exposed tryptophanyl residue in a protein at a wavelength of 300 nm or greater and collection of the fluorescence at a wavelength greater than 380 nm should make contributions from level kinetics negligible in anisotropy measurements and thus reveal rapid internal motions.

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