Oxygen Quenching and Fluorescence Depolarization of Tyrosine Residues in Proteins*

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We examined the dynamics of oxygen quenching and depolarization of tyrosine fluorescence in small peptides and proteins lacking tryptophan. The oxygen-quenching constants and the apparent correlation times for fluorescence depolarization were found to be sensitive to the conformational state of the proteins. For small peptides and random coil proteins, the oxygen bimolecular quenching constants indicated complete accessibility of the tyrosine residues to collisions with oxygen. For folded proteins, the quenching constants were about 2-fold smaller, indicating only limited shielding of the tyrosine residues from oxygen by the protein matrix. We also used the steady state anisotropies, measured under conditions of oxygen quenching, to estimate the motional freedom of the tyrosine residues. For random coil proteins, such as a tyrosine copolymer and histones at low pH, the data clearly indicated that depolarization occurs due to subnanosecond segmental motions of the tyrosine residues which are independent of overall protein rotation. For some folded proteins, including bovine pancreatic trypsin inhibitor, the data are consistent with, but do not unambiguously demonstrate, motional freedom of the residues. In these cases, energy transfer among tyrosine residues may also contribute to the observed depolarization. Overall, these results indicate that the rate and extent of tyrosine rotation in proteins depend upon the conformation of the protein and the specific protein under observation.

The functional properties of proteins may be dependent upon their dynamic properties on time scales ranging from seconds to picoseconds (1, 2). The extensive theoretical and experimental studies of protein dynamics have been recently reviewed (3, 4). Fluorescence spectroscopy has been particularly useful in the study of the dynamics of macromolecules primarily because of its natural time window on the nanosecond time scale (5). To date, fluorescence methods have revealed the dynamic properties of proteins by oxygen and acrylamide quenching (6, 7), time-resolved and lifetime-resolved measurements of tryptophan depolarization (8–10), and wavelength-resolved lifetimes (11). The experimental data published to date were obtained primarily for the fluorescence from tryptophan residues in proteins. This is because the quantum yields of tryptophan fluorescence from proteins are significantly higher than those of tyrosine fluorescence. Also, tyrosine fluorescence cannot be accurately quantified in the presence of tryptophan because the overlapping tryptophan emission is dominant and because of substantial energy transfer from tyrosine to tryptophan.

On the other hand, it is of interest to experimentally quantify the dynamics of tyrosine residues so that its behavior can be compared with that of tryptophan. Also, such data can be compared with the extensive theoretical calculations of protein dynamics (12–14) and with the experimental results obtained by nuclear magnetic resonance methods (15–18). In this paper, we describe measurements of the rotational motions of tyrosine residues in peptides and proteins. We measured the steady state fluorescence anisotropies as the fluorescence lifetimes were decreased by oxygen quenching (9, 10, 19). Such measurements yield estimates of the rotational correlation time of the fluorescent residue. Under favorable circumstances, one can infer whether there exist segmental motions which occur on the subnanosecond time scale. We examined a range of peptides, starting with tyrosine and small peptides, and progressing to insulin, BPTI, ribonuclease A, and histones H1, H2A, and H3. For the RNase A and the histones, we compared the dynamic properties of the tyrosine residues when these proteins were in the folded and the random coil states.

**THEORY**

Dynamic or collisional quenching of fluorescence by oxygen can be described by the Stern-Volmer equation

\[ \frac{F_0}{F} = 1 + k_q \tau_o [O_2] \]

where \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of oxygen, respectively, \(\tau_o\) is the fluorescence lifetime in the absence of quenching, and \(k_q\) is the bimolecular quenching constant. The value of \(k_q\) is of interest because it reflects the collisional frequency of the fluorophore with oxygen, and hence the extent to which the tyrosine residues are shielded from such collisions by the surrounding protein matrix. For an efficient quencher such as oxygen, the expected value of \(k_q\) is given by

\[ k_q = 4 \pi R D N / 1000 \]

where \(R\) is the sum of the molecular radii of the fluorophore and quencher, \(D\) is the sum of their diffusion coefficients, and \(N\) is Avogadro’s number. The Stern-Volmer quenching con-

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1 The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; NATyrA, N-acetylstearoyl-t-tyrosinamide; Tyr-Tyr, tyrosyl-tyrosine; Leu-Tyr-Leu, leucyl-tyrosyl-leucine; enkephalin or [Leu5]enkephalin, Tyr-Gly-Gly-Phe-Leu; Glu-Ala-Tyr, co-polymer of glutamate, alamine, and tyrosine with a molar ratio of 65:26:9; 2-HFP, 2-hexafluoropropanol.
stant \( K = k_s r_0 \) is determined by a plot of \( F_0/F \) versus [O\(_2\)]. The independently determined lifetime \( (\tau_0) \) is then used to calculate \( k_s \). For quenching of small molecules by oxygen in aqueous solution at 25 °C, values of \( k_s \) are typically near \( 1.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \) (20). Observation of this value indicates that the fluorophore is exposed to the aqueous phase, while smaller values of \( k_s \) indicate shielding of the residue from collisions with oxygen. Hence, for tyrosine residues which are within a protein matrix, the measured value of \( k_s \) indicates the rate at which molecular oxygen penetrates the protein.

A second useful feature of collisional quenching is that the fluorescence lifetime is decreased in proportion to the fluorescence yield. That is, the lifetime in the presence of oxygen quenching \( (\tau) \) is given by

\[
\tau = \tau_0 \frac{F}{F_0}
\]

The lifetime of the quenched sample can be calculated from the unquenched lifetime \( (\tau_0) \) and the decrease in yield caused by quenching \( (F/F_0) \). Consider a tyrosine residue which is bound rigidly within a protein whose rotational correlation time is \( \phi \). The steady state anisotropy of the tyrosine fluorescence is given by

\[
r = \frac{r_0}{1 + \tau/\phi}
\]

where \( r \) and \( r_0 \) are the anisotropies in the presence and absence of rotational diffusion, respectively. Generally, \( r_0 \) values are measured in the absence of rotational diffusion by the use of low temperature and vitrifying solvents. For proteins in fluid solution, a decrease in lifetime results in less time for rotational diffusion and hence higher anisotropies. The correlation time of the motion can be estimated from a plot of \( r^{-1} \) versus \( \tau \). This is apparent from a rearrangement of Equation 4,

\[
\frac{1}{r} = \frac{1}{r_0} + \frac{\tau}{\phi r_0}
\]

One expects the \( y \) intercept to be \( r_0^{-1} \) and the slope to be \( (\tau/\phi) r_0 \). The appearance of lifetime-resolved anisotropy data expected for tyrosine residues in proteins is illustrated by the model calculations shown in Fig. 1. For tyrosine residues, the typical range of lifetimes accessible by oxygen quenching is 0.6 to 1.8 ns. This is the region illustrated by solid lines. For the rotational correlation time, we chose \( \phi = 6 \text{ ns} \), which is comparable to that expected for RNase A in aqueous solution at 25 °C. The limiting anisotropy \( (r_0) \) was chosen to be 0.29, which is close to the value observed for tyrosine residues using our experimental conditions. From Fig. 1A (with \( \alpha = 0 \)), one can see that, upon quenching the fluorescence lifetime from 1.8 to 0.5 ns, one expects an increase in the anisotropy from 0.232 to 0.267 ns. The \( y \) intercept will be equal to \( r_0^{-1} \). Given the precision of steady state anisotropy measurements, these calculations indicate that correlation times in the nanosecond range may be accurately measured using this method.

The analysis of lifetime-resolved anisotropies becomes more complex and ambiguous for a fluorophore which has motional freedom within the protein. For a fluorophore which rotates within a protein, the time-resolved anisotropy is approximately given by

\[
r(\tau) = r_0 e^{-\tau/\phi} [ar^{-1/2} + (1 - a)]
\]

where \( \phi \) is the correlation time for the internal motion and \( a \) is the fraction of the total anisotropy which decays as a result of the internal motion. In this simple model, we assumed that the decays of anisotropy due to both the overall protein rotation and to the segmental motion are single exponentials, and that these motions are independent from each other. The fractional amplitude of the segmental motion can be approximated by

\[
1 - a = \left( \frac{3 \cos \theta - 1}{2} \right) = \frac{r(0)}{r_0}
\]

where \( \theta \) is a measure of the angular displacement of the tyrosine residue due to the segmental motion. To be precise, \( \cos \theta \) is the average value of this parameter at times longer than \( \phi \), but shorter than \( \phi_p \), that is, the average value of \( \cos \theta \) due to segmental motion. The extent of depolarization due to the rapid segmental motion can also be expressed by \( r(0)/r_0 \), where \( r(0) \) is understood to be the anisotropy at \( t = \tau \), and \( \phi = 54.7° \) corresponds to a residue with completely free motion within the protein \( (\alpha = 1) \). For completeness, we note that other definitions have been used to describe independent motions of fluorophores. For example, \( \phi \) can be related to the angle \( (\phi) \) at which the residue strikes a barrier beyond which it cannot rotate by \( (1 - a) = [(1/2) \cos \theta, (1 + \cos \theta)\phi] \) (8, 34, 35). Using this model, \( \phi \) corresponds to \( \alpha = 0 \), and \( \phi = 90° \) corresponds to \( \alpha = 1 \). We note that this expression is strictly true only when the absorption and emission oscillators are co-linear. More complex expressions are needed when these oscillators are not co-linear, that is, when \( r_0 < 0.40 \) (34, 35). We will use Equation 7 as our basis for estimating the angular displacements of tyrosine residues due to segmental motions.

In the lifetime domain, Equation 6 becomes

\[
r = \frac{ar_0}{1 + \left[ \frac{1}{\phi_0} + \frac{1}{\phi_0} \right]^{-1} + (1 - a)r_0}
\]

From this equation, and Equation 6, one sees that the fraction of the total anisotropy which decays by overall protein rotation is given by \( r(0)/r_0 = 1 - a \). A typical correlation time for the segmental motion, as determined from the molecular dynamics calculations, is 2 ps. Using \( \theta = 30° \), a typical value for the torsional displacement of tyrosine residues obtained
from the molecular dynamics calculations on BPTI, one obtains $\alpha = 0.37$ (12). Substitution of $\phi_T = 2$ ps and $\phi_R = 6$ ns into Equation 8 yields the simulated data shown in Fig. 1A. If a 2-ps motion were present with $\alpha = 0.37$, then the anisotropy extrapolated to $r = 0$ is expected to be 37% smaller than $r_0$ (Fig. 1A). The rapid motion would be revealed by an increase in the y intercept. Since the time resolution of our experiments do not extend to 2 ps, the time constant for such rapid motions cannot be determined. Nonetheless, the existence of such rapid motions may be inferred from comparison of $r(0)$ with $r_0$. The value $\alpha = 0.37$ probably overestimates the loss of anisotropy due to torsional motions by about a factor of 2.

Levy and Szabo (14) calculated the initial loss in anisotropy due to torsional motions of the tyrosine residues in BPTI and found an average value of $\alpha = 0.13$. If the fractional loss of anisotropy due to the rapid motion is near 0.13, then $r(0)$ is expected to be 13% smaller than $r_0$ (Fig. 1A). Following this initial rapid loss of anisotropy, the remaining anisotropy ($r_0(1 - \alpha)$) would decay exponentially with a correlation time of 6 ns. In our experiments, such an exponential decay is equivalent to a linear dependence of $r$ versus $\tau$.

The presence of an internal motion with a correlation time of more than 100 ps is more difficult to quantify because such a motion alters both the slope and the y intercept of the plot of $r-l$ versus $\tau$ (Fig. 1B). For instance, values of $\alpha = 0.50$ and $\phi_T = 0.2$ ns would result in a decrease in $r(0)$ from 0.290 to 0.189 and in a decrease in the apparent correlation time from 6 to 3.6 ns. The apparent correlation time ($\phi_A$) is obtained from the slope of $r^{-1}$ versus $\tau$ using the available data, which is indicated by the solid lines. Hence, rapid internal motions can be revealed by a decrease in $r(0)$, a decrease in $\phi_A$, or both. Due to practical limitations in dissolving oxygen in aqueous solutions, we cannot decrease the lifetime to the region of curvature and, hence, we cannot determine $\alpha$ and $\phi_T$ in an unambiguous manner.

In the above discussion, we assumed that the tyrosine fluorescence could be described by a single fluorescence lifetime. While this is probably true for peptides containing a single tyrosine residue, multiple lifetimes are likely for proteins containing more than a single residue. Presently, it is not possible to determine the individual lifetimes and quenching constants, as would be needed to determine the individual correlation times of each residue in a protein. However, this heterogeneity seems unlikely to affect our overall conclusions. For example, if a tyrosine residue with a short lifetime is present, its relative quantum yield and contribution to the measured anisotropy is also likely to be small. For these reasons, we have not attempted to separately determine the lifetimes and correlation times of individual tyrosine residues where multiple residues were present in a given protein.

The expected values for the overall rotational correlation times of the proteins can be estimated using

$$\phi_R = \frac{f \eta V}{\mu T}$$

where $\eta$ is the viscosity, $V$ is the volume of the anhydrous protein, $\mu$ is the gas constant, and $f$ is a shape and hydration factor. Typically, the rotational correlation times of proteins are well approximated by Equation 9 with $f$ = 1.5 to 2 (10). The latter value will be used in our calculations.

**MATERIALS AND METHODS**

BPTI was a gift from C. Woodward (University of Minnesota). NATyrA and tyrosine were obtained from Aldrich. Leu-Tyr-Leu-Leu was purchased from Research Plus Chemicals and Tyr-Tyr, Leu-enkephalin, oxytocin, histones H3, H2A, and H1, the copolymer Glu-Ala-Tyr (44, 50,000), bovine insulin, and RNase A were from Sigma Chemical Co.

**RESULTS**

**Fluorescence Spectra of Tyrosine Residues**—The uncorrected excitation and emission spectra of NATyrA are shown in Fig. 2. The emission is not greatly displaced from the excitation, that is, the Stokes shift for tyrosine fluorescence is small. This fact, and the weak tyrosine fluorescence which is often found for folded proteins, results in the need for caution so as to avoid extraneous signals from either background fluorescence or scattered light from the sample. Also shown in Fig. 2 is the spectrum of a blank buffer solution, recorded at the same amplification needed for the emission spectrum of BPTI. Among the proteins we examined, BPTI showed the weakest intensity, and hence required the largest amplification. For the blank solution, the peak near 316 nm is due to Raman scatter from the water. To avoid this scattered light, and to provide maximum sensitivity, we chose to measure the tyrosine fluorescence through an interference filter with a
ground values were found to be less than 2% of the total previously for oxygen quenching of protein fluorescence in lifetimes, allow calculation of the bimolecular quenching constants. These values are summarized in Table I. For tyrosine, ground fluorescence is essential for reliable measurements of fluorescence. Careful elimination of scattered light and background fluorescence is essential for reliable measurements of tyrosine fluorescence and anisotropy.

**Oxygen-quenching Constants for Tyrosine Fluorescence**—Typical Stern-Volmer plots for tyrosine fluorescence from peptides and proteins are shown in Figs. 3 and 4. As was found previously for oxygen quenching of protein fluorescence in aqueous solution (6, 20), the plots are linear, indicating the absence of static quenching. These data, and the measured lifetimes, allow calculation of the bimolecular quenching constants. These values are summarized in Table I. For tyrosine, NATyrA, and the small tyrosine-containing peptides, the values of $k_q$ are all near $1.2 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$. This value indicates that the tyrosine residues are completely accessible to quenching by oxygen. For the histones at low pH and the tyrosine co-polymer at pH 7.5, where these proteins exist as random coils, the values of $k_q$ are also near $1 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$, which again indicates the accessibility of these residues to oxygen.

The histones exist as random coils at acidic pH. Upon addition of salts at neutral pH, a more compact structure is formed. For histones H2A and H3, 3.2 mM phosphate induces the formation of about 15-20% α-helix and some β-sheet. Similarly, addition of 30 mM neutral phosphate buffer to histone H1 results in a more compact structure (26). Some of the tyrosine residues become buried in the folded regions (23, 24). For histones H2A and H3, the extent of secondary structure can be further increased by solvents of low polarity such as polyhydroxy alcohols and 2-HPF (25). There was some decrease in the bimolecular-quenching constant for these histones due to folding, indicating that at least some of the tyrosine residues are partially shielded from collisions with oxygen. In a similar manner, the bimolecular quenching constants of RNase A, insulin, and BPTI are about 1.5- to 2-fold smaller than those expected for diffusion-controlled quenching. These results also suggest some shielding of tyrosine residues by the protein matrices. However, it is not possible to interpret these results in terms of permeation of protein matrix by oxygen in an unambiguous manner. This is because the fluorescence may result from tyrosine residues which are on or near the surfaces of these proteins (27-29).

It is interesting to examine the lifetimes of tyrosine in the various solutions listed in Table I. The lifetimes of tyrosine and NATyrA are both smaller in phosphate buffer than in Tris buffer, even though the pH values are identical. The $pK_a$ of the phenolic hydroxyl group is near 10. Upon excitation, the $pK_a$ decreases to about 2. Phosphate is more effective than Tris in removing the phenolic proton. Since tyrosinate is only weakly fluorescent, this ionization effectively quenches the emission from tyrosine. Hence, the lifetimes are smaller in the phosphate buffer. Likewise, the lifetime of the polymer Glu-Ala-Tyr (65:26:9) is short because of the quenching effects of the carboxylate groups.

For examination of the anisotropy decay times from the lifetime-resolved anisotropies, a wide range of quenched lifetimes is desirable. This range is determined by the bimolecular quenching constant $k_q$ and the unquenched lifetime $\tau_0$. In favorable circumstances, such as for NATyrA, approximately a 7-fold range of lifetimes is available (Fig. 3). However, in other cases, such as RNase A, insulin, and BPTI (Fig. 4), this range is only 2-fold or smaller. In the case of BPTI, the lifetime can only be decreased 30% by quenching, due primarily to its short unquenched lifetime (0.35 ns). This unfortunate circumstance limits the comparison of our data with the theoretical calculations of protein dynamics for BPTI.

**Limiting Anisotropies of Tyrosine Residues**—Analysis of our data requires knowledge of the limiting anisotropy in the absence of rotational diffusion ($r_0$). These values were determined at $-62^\circ\text{C}$ in 70% propylene glycol (Table II). Similar values were observed among the tyrosine, NATyrA, Leu-Tyr-Leu, enkephalin, oxytocin, and histone H1. The average of these values was 0.279 at 285 nm. Each of these substances...
TABLE I

<table>
<thead>
<tr>
<th>Substance</th>
<th>Buffer</th>
<th>°C</th>
<th>τ</th>
<th>K</th>
<th>$k_i \times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>0.02 M Tris</td>
<td>25</td>
<td>3.4</td>
<td>4.5</td>
<td>1.3</td>
</tr>
<tr>
<td>0.05 M phosph</td>
<td>25</td>
<td>2.1</td>
<td>27</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>NATyrA</td>
<td>0.02 M Tris</td>
<td>25</td>
<td>1.6</td>
<td>22</td>
<td>1.4</td>
</tr>
<tr>
<td>0.05 M phosph</td>
<td>25</td>
<td>1.3</td>
<td>17</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Tyr-Tyr</td>
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<td>25</td>
<td>2.0</td>
<td>24</td>
<td>1.2</td>
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<tr>
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<td>0.05 M phosph</td>
<td>25</td>
<td>1.8</td>
<td>20</td>
<td>1.1</td>
</tr>
<tr>
<td>[Leu' furyl-phenyl (Tyr-Gly-Gly-Phe-Leu)]</td>
<td>0.05 M phosph</td>
<td>25</td>
<td>1.4</td>
<td>17</td>
<td>1.2</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>0.05 M phosph</td>
<td>25</td>
<td>1.0</td>
<td>8</td>
<td>0.9</td>
</tr>
<tr>
<td>Glu-Ala-Tyr (65:26:9)</td>
<td>0.05 M phosph</td>
<td>25</td>
<td>0.8</td>
<td>10</td>
<td>1.2</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.01 M Tris +</td>
<td>25</td>
<td>1.0</td>
<td>6</td>
<td>0.6</td>
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<tr>
<td>0.001 M NaCl, pH 8.0</td>
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<td>1.3</td>
<td>8</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>RNase A</td>
<td>0.05 M phosph</td>
<td>25</td>
<td>1.1</td>
<td>18</td>
<td>0.7</td>
</tr>
<tr>
<td>+0.1 M KCl, pH 1.3</td>
<td>25</td>
<td>1.1</td>
<td>8</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>BPTI</td>
<td>0.05 M phosph</td>
<td>25</td>
<td>0.55</td>
<td>3</td>
<td>0.8</td>
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<tr>
<td>Histone H3</td>
<td>pH 3</td>
<td>25</td>
<td>1.5</td>
<td>15</td>
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</tr>
<tr>
<td>3.2 mm phosph</td>
<td>25</td>
<td>1.5</td>
<td>10</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>3.2 mm phosph</td>
<td>4</td>
<td>1.6</td>
<td>6</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>3.2 mm phosph +</td>
<td>4</td>
<td>1.8</td>
<td>8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2% DMF</td>
<td>32 mm phosph</td>
<td>25</td>
<td>1.5</td>
<td>11</td>
<td>0.8</td>
</tr>
<tr>
<td>pH 3</td>
<td>4</td>
<td>1.7</td>
<td>11</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>3.2 mm phosph</td>
<td>4</td>
<td>1.8</td>
<td>7</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>3.2 mm phosph +</td>
<td>4</td>
<td>2.0</td>
<td>8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>2% DMF</td>
<td>30 mm phosph</td>
<td>25</td>
<td>1.8</td>
<td>11</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The pH of the 0.02 M Tris and 0.05 M phosphate (phos) buffers was 7.5.
*The pH of the histones solutions in 3.2 and 30 mM phosphate was 7.0.
*At 5°C, the value of $k_i$ expected for an exposed residue is near 8.0 x 10⁻³ M⁻¹ s⁻¹.

Contains a single tyrosine residue. Evidently, the neighboring groups have little effect on $r_o$. These values are comparable to those reported previously for tyrosine (30). Somewhat smaller $r_o$ values were found for the other compounds, ranging from 0.204 for Tyr-Tyr to 0.281 for RNase A. The smaller values of $r_o$ are probably due to energy transfer among the tyrosine residues (30, 31). Unfortunately, it is difficult to quantify the extent of energy transfer in proteins and the extent of depolarization caused by this process. In the case of the histones, the propylene glycol induces significant secondary structure (26), which probably facilitates energy transfer among the tyrosine residues. This is evident from an increase in $r_o$ when histones H1 and H2A were dissolved in 10 M guanidine hydrochloride in place of buffer and then mixed with the propylene glycol. Hence, the $r_o$ values for histones H1 and H2A may be smaller than the actual values in aqueous solution due to enhanced energy transfer. We will use the larger $r_o$ values for our estimates of the angular range of the segmental motions.

Apparent Correlation Times for Depolarization of Tyrosine in Peptides—The lifetime-resolved anisotropies for tyrosine and other small peptides are shown in Figs. 5 and 6. From the slopes of the $r$ versus $τ$ plots, we estimated the apparent correlation times for fluorescence depolarization (Table III). For tyrosine and NATyrA, the measured correlation times are near 35 ps. As expected, the measured values are independent of the buffer and the unquenched lifetimes. The calculated values of the correlation time ($φ_k$) are also listed in Table III. For tyrosine and NATyrA, the measured values are severalfold smaller than those calculated from Equation 9. A similar observation was made for N-acetyl-tryptophanamide (10) and for small fluorophores in solution (32). The phenol ring can probably rotate independently of the backbone of the amino acid, and because it is comparable in size to the solvent, this rotation can be more rapid than predicted by the Stokes-Einstein equation. Interestingly, a similar but somewhat smaller discrepancy was found for the dipeptide Tyr-Tyr (Table III), where significant energy transfer is thought to occur between the two tyrosine residues (30).
Energy transfer is probably responsible for the smaller value of $r_0$ (Table II), and it is important to consider the effect of energy transfer on the apparent correlation times. Such transfer may be expected to increase the rate of depolarization and hence decrease the apparent correlation times. Apparently, only a limited number of transfers occur, and the time scale of these transfers results in a decrease in $r_0$ but not in the measured correlation times.

Also listed in Table III are the measured and calculated correlation times for several small peptides. The tripeptide Leu-Tyr-Leu displays a 3-fold larger correlation time than tyrosine or NATyrA. In Leu-Enkephalin (Tyr-Gly-Gly-Phe-Val), the effect of energy transfer is probably responsible for the smaller value of $r_0$ (Table II), and it is important to consider the effect of energy transfer on the apparent correlation times. Apparently, only a limited number of transfers occur, and the time scale of these transfers results in a decrease in $r_0$ but not in the measured correlation times.

TABLE III
Rotational correlation times and $r(0)$ values for tyrosine-containing peptides and proteins

<table>
<thead>
<tr>
<th>Substance</th>
<th>Buffer</th>
<th>$^\circ$C</th>
<th>$\phi_0$</th>
<th>$\phi_v$</th>
<th>$r(0)$</th>
<th>$r(0)/r_0$</th>
<th>$\theta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>0.02 M Tris*</td>
<td>25</td>
<td>0.032</td>
<td>0.115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05 M phos</td>
<td>25</td>
<td>0.032</td>
<td>0.115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NATyrA</td>
<td>0.02 M Tris</td>
<td>25</td>
<td>0.037</td>
<td>0.117</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06 M phos</td>
<td>25</td>
<td>0.037</td>
<td>0.117</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tyr-Tyr</td>
<td>0.05 M phos</td>
<td>25</td>
<td>0.077</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Leu-Tyr-Leu</td>
<td>0.05 M phos</td>
<td>25</td>
<td>0.10</td>
<td>0.22</td>
<td></td>
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<tr>
<td>[Leu]enkephalin</td>
<td>0.05 M phos</td>
<td>25</td>
<td>0.18</td>
<td>0.33</td>
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<tr>
<td>Oxytocin</td>
<td>0.06 M phos</td>
<td>25</td>
<td>0.40</td>
<td>0.53</td>
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<tr>
<td>Glu-Ala-Tyr</td>
<td>0.05 M phos</td>
<td>25</td>
<td>0.53</td>
<td>27.0</td>
<td>0.182</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0.01 M Tris + 0.1 M NaCl, pH 8</td>
<td>25</td>
<td>2.0</td>
<td>18.5</td>
<td>0.190</td>
<td>0.80</td>
<td>21</td>
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<tr>
<td>RNase A</td>
<td>0.05 M phos</td>
<td>25</td>
<td>4.4</td>
<td>7.2</td>
<td>0.263</td>
<td>0.94</td>
<td>12</td>
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<tr>
<td>pH 1.3 + 0.1 M KCl</td>
<td>25</td>
<td>2.7</td>
<td>7.2</td>
<td>0.217</td>
<td>0.81</td>
<td>21</td>
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<tr>
<td>BPTI</td>
<td>0.05 M phos</td>
<td>25</td>
<td>2.7</td>
<td>3.4</td>
<td>0.260</td>
<td>1.05</td>
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<tr>
<td>Histone H3</td>
<td>pH 3</td>
<td>25</td>
<td>1.0</td>
<td>8.1</td>
<td>0.167</td>
<td>0.71</td>
<td>26</td>
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<tr>
<td>3.2 mm phos</td>
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<td>3.6</td>
<td>8.1</td>
<td>0.245</td>
<td>1.03</td>
<td>0</td>
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<tr>
<td>3.2 mm phos + 2% HFP</td>
<td>4</td>
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<td>15</td>
<td>0.257</td>
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<tr>
<td>Histone H2A</td>
<td>pH 3</td>
<td>25</td>
<td>1.0</td>
<td>7.4</td>
<td>0.167</td>
<td>0.62</td>
<td>30</td>
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<tr>
<td>3.2 mm phos</td>
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<td>3.8</td>
<td>7.4</td>
<td>0.208</td>
<td>0.78</td>
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<tr>
<td>pH 3</td>
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<td>1.4</td>
<td>14</td>
<td>0.182</td>
<td>0.68</td>
<td>28</td>
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<tr>
<td>3.2 mm phos + 2% HFP</td>
<td>4</td>
<td>5.2</td>
<td>14</td>
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<td>Histone H1</td>
<td>pH 3</td>
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<td>4.4</td>
<td>14</td>
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<td>10.6</td>
<td>0.154</td>
<td>0.60</td>
<td>31</td>
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</table>

* The pH of the 0.02 M Tris and 0.05 M phosphate (phos) buffers was 7.5.
+ RNase A may not be completely denatured at pH 1.3, but the structure seems to be expanded (40).
Leu), the tyrosine correlation time was 0.18 ns, while for oxytocin it was 0.40 ns. Oxytocin is a nanopeptide. A ring formed by a disulfide bridge contains 6 amino acid residues which includes the tyrosine residue. The correlation times for enkephalin and oxytocin obtained by fluorescence depolarization are in excellent agreement with those obtained by NMR (17, 33). The apparent tyrosine correlation time for the Glu-Ala-Tyr co-polymer is substantially smaller than that calculated on the basis of its average molecular weight, as may be expected for a random coil polymer. For the peptides shown in Figs. 5 and 6, the largest measured anisotropies, at the shortest quenched lifetimes, are considerably smaller than the known values of ro. Consequently, one cannot extrapolate with any confidence to \(\tau = 0\), and hence, one cannot estimate the amplitude of any faster unresolved motions. Such motions, although likely to be present in these peptides, cannot be resolved using our method.

**Depolarization of Tyrosine Residues in Proteins—Lifetime-resolved anisotropies for the tyrosine residues in proteins and co-polymers are shown in Figs. 7-9. The data are summarized in Table III.** The steady state anisotropies of the histones, and the magnitude of the effect of phosphate on the anisotropies, are similar to those reported by Isenberg and co-workers (23, 24, 26). First we note the effects of the random coil conformation on tyrosine dynamics. For the histones, the apparent correlation times are about 1 ns, which are significantly smaller than those expected for overall protein rotation (Table III). We previously found similar results for tryptophan residues in proteins in the presence of 6 M guanidine hydrochloride (10). In that case, the correlation times were near 2 ns, which probably reflects the larger size of the indole ring as compared to the phenol ring. These correlation times for the random coil structures suggest that depolarization is primarily due to large scale segmental motions and internal rotations. We further note that similar results were found for histone H1 (Fig. 9) and histones H3 and H2A (Fig. 8). The former contains only a single tyrosine residue (26), whereas the others each contain several such residues. The similar results suggest that tyrosine-tyrosine energy transfer did not seriously alter the apparent correlation times or that energy transfer is not significant in the random coil conformations.

For each of the histones at low pH, the \(\tau = 0\) intercepts indicate considerable freedom for motions with correlation times shorter than the quenched lifetimes. The decay times for the more rapid motions cannot be determined from our measurements because of our inability to shorten the lifetimes to the curved region of the \(r^{-1}\) versus \(\tau\) plots. For the three histones, the average value of \(r(0)/r_o\) is 0.64. This value corresponds to a displacement of 30° (Equation 7) at times shorter than 0.5 ns. Hence, the tyrosine residues in random coil proteins display considerable motional freedom. There was an increase in both apparent correlation times and the \(r(0)\) values due to folding of histones in the presence of phosphate and HFP. This suggests a restriction of motional freedom of tyrosine residues due to protein folding. Similar behavior has been observed by proton NMR for histones H3 and H2A (36, 37).

At neutral pH, the results for RNase A indicate the presence of only small amplitude motions. There is an increase in these motions at pH 1.3, as suggested by the decrease in \(r(0)\) and the apparent correlation time. These results suggest that RNase A, at acidic pH at room temperature, is more like a
swollen protein than like a random coil, and are in agreement with earlier spectroscopic, NMR relaxation, and hydrodynamic studies (38-40). In contrast, the apparent correlation time and \( r(0) \) values for insulin suggest the presence of significant segmental freedom for the tyrosine residues. However, in comparison to the large segmental motions observed in random coils, these motions are significantly restricted.

**DISCUSSION**

Lifetime-resolved anisotropies were measured for a number of tyrosine-containing peptides and proteins. The results for the small peptides indicate that faster motions can be detected if their amplitudes exceed about 15°, corresponding to a fractional depolarization of 10%. Our results demonstrate that the dynamic properties of tyrosine residues are dependent upon the conformational state of the proteins. In general, the tyrosine correlation times in the random coil state are near 1 ns, and the data indicate the existence of faster motions through a cone angle of 30°.

One aim of this investigation was to compare experimental studies of fluorescence depolarization with the molecular dynamic calculations of depolarization by Levy and Szabo (14). For BPTI, these workers calculated that a small degree of depolarization (\( \alpha = 0.13 \)) would occur in 2 ps. Such rapid motions of this amplitude would decrease the apparent \( r(0) \) values by 13%. This decrease in \( r(0) \) is comparable to the variability of the \( r(0) \) values among different proteins, the uncertainty in estimation of this value from the vitrified solution data, and the uncertainties due to energy transfer among the tyrosine residues. Furthermore, the unquenched lifetime of BPTI is 0.35 ns. As a result only a small range of lifetimes could be observed by quenching, (0.25 to 0.35 ns). Our data are consistent with rapid motions of this amplitude. However, because of the reasons listed above, our experiments were not adequate to verify or refute the molecular dynamics calculations on BPTI.

**REFERENCES**

Oxygen quenching and fluorescence depolarization of tyrosine residues in proteins.
J R Lakowicz and B P Maliwal


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