Internal Dynamics and Overall Motion of Lysozyme Studied by Fluorescence Depolarization of the Eosin Lysozyme Complex

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Abstract

Time-resolved fluorescence depolarization on the nanosecond and sub-nanosecond time scales is a powerful technique for the study of rapid motions in the condensed phase. We apply this technique to measure the motions of proteins using both extrinsic and intrinsic probes. Eosin, which absorbs and fluoresces in the visible, forms a one-to-one complex with lysozyme binding in the hydrophobic box region and is used as an extrinsic probe of lysozyme motion. The long-time anisotropy of bound eosin is used to measure the overall rotation time of lysozyme for which refined values are presented. In addition, our measurements show a rapid restricted motion of the eosin molecule on the time scale of ~100 ps. The order parameter, a model independent measure of the extent of the restriction of the rapid motions, decreases with increasing temperature, indicating that the motion of the eosin is less hindered as temperature increases. We compare our results with the crystallographic measurements of least square displacements for the hydrophobic box region. Our measurements provide direct time resolved confirmation that the displacements observed in this region correspond to rapid motion.

Introduction

The dynamic structure of proteins has become a topic of considerable interest over the last few years. Evidence has emerged from x-ray crystallographic studies, NMR experiments, and molecular dynamics calculations for the existence of rapid motion in proteins. To complement these approaches it will be very valuable to have time resolved measurements which are capable of revealing the motion directly. Fluorescence techniques have considerable potential for studies of this type; the fluorescence depolarization of tryptophyl residues has been studied by time correlated single photon counting with subnanosecond time resolution, and by steady state fluorescence quenching.

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Time resolved measurements of the anisotropy, \( r(t) \), provide a number of different types of information. If the molecular system undergoes a diffusive tumbling, the long time behavior of \( r(t) \) is an exponential decay which describes the overall reorientation of the probed molecule; if the decay in the shorter time region deviates from that extrapolated back to zero time from this asymptotic behavior, it can be (but is not always, see below) an indication of the presence of a rapid restricted motion. A quantitative and model independent measure of the extent to which these restricted motions occur is given by the order parameter,\(^{14,15} S^2 \). The experimentally measured value of the initial anisotropy, \( r(0) \), also provides an indicator of the presence of rapid processes including motion which has occurred at times shorter than the time resolution of the experiment when it is less than the theoretically predicted value, 0.4. Very precise data are required to extract accurate values of these parameters from polarized fluorescence curves and we have recently described our own analysis procedure and the serious pitfalls associated with these experiments in some detail.\(^{16} \) A number of other commonly used analysis procedures can lead to anisotropy parameters widely at variance with the correct values if, for example, convolution of the measured curves is not taken into account properly, or if the curves are only fit for times longer than that determined by the position of the peak channel.

Once the anisotropy is obtained, the interpretation may not be straightforward. This seems to be the case with tryptophan since the theoretical limiting anisotropy (i.e., \( r(0) = 0.4 \)) is never obtained.\(^{12,17-18} \) In cases where electronic (or possibly vibrational) relaxation occurs on a time scale equal to or shorter than that of the anisotropy measurement, the presence of a short component in \( r(t) \) or a measured value of \( r(0) < 0.4 \) does not necessarily indicate rapid motion.

We recently completed a theoretical study of the effect of excited state kinetics and reorientational motion on time-resolved absorption and emission experiments,\(^{19} \) providing a general algorithm for calculating the signals observed in these experiments on systems with many communicating levels. Since such experiments are effected by both electronic relaxation and molecular reorientation, the observed signals can have complicated forms which represent coupling of these two effects in a way that depends on the particular scheme of decay processes which are present in a given system. Among the other systems studied in this work was the fluorescence of tryptophan, which is complicated by the overlap of \( ^1L_a \) and \( ^1L_b \) absorption spectra in the 250-350 nm region. We found that the form of the anisotropy decay depends strongly on the ratio of excited states prepared, and consists generally of an initial rapid decay corresponding to attaining a quasi-equilibrium between the two excited states, and a slower decay corresponding to the motion of the chromophore. Even if excitation occurs at long enough wavelengths to excite the lower lying state, the anisotropy can apparently decrease as the upper state is thermally populated. Here the existence of a short component, or a low measured \( r(0) \) value in a fluorescence anisotropy measurement does not unambiguously indicate rapid motion.
The possible complications arising from tryptophan are avoided in this work by using an intrinsic probe (eosin) to study the dynamics of lysozyme. The photo-physics of eosin is well characterized, and in two previous experiments we measured $r(0) = 0.40 \pm 0.02$, in agreement with the theoretically limiting value, and found that both $r(t)$ and $K(t)$ (the fluorescence decay) were described well by single exponential decays. Grossweiner and coworkers have shown that eosin forms a 1:1 complex with lysozyme and the binding site is in the hydrophobic box region. The eosin molecule can be excited directly with visible light (without any excitation of the tryptophyl residues), and the fluorescence anisotropy should serve as a probe both of the motions associated with the residues that form the hydrophobic box and of the overall protein motion.

**Time-Resolved Anisotropy Measurements.**

Time-resolved fluorescence emission curves were measured by time-correlated single photon counting. The excitation source consisted of an actively mode locked argon ion laser (Coherent CR-6) operating at a repetition rate of 93.54 MHz and a wavelength of 514.5 nm. The repetition rate of the source was reduced by a low-voltage electrooptic modulator (Coherent Model 28) driven by a countdown logic circuit synchronized to the mode-locker rf source, selecting pulses at a rate of 91.3 kHz. The contrast ratio between selected and rejected pulses was 100:1. The “start” signal for the time-to-amplitude converter (ORTEC 457) was obtained from a constant fraction discriminator (ORTEC 583) which had as input a signal from a photodiode (Telefunken BPW28) monitoring the selected pulse train. Fluorescence emission detected at right angles to the excitation was detected by a photomultiplier (Philips XP2020Q) after passing through filters which absorbed the scattered excitation light, and through a polarizing element (Polaroid HNP,B Plastic) with orientation either parallel, perpendicular, or at the magic angle relative to the polarization of the excitation pulse. The polarizer was mounted on a rotation stage (NRC RSA-2) which could be positioned to an accuracy of about 0.5°. The photomultiplier output was amplified (ENI 600L) and sent to a second discriminator, which generated the “stop” signal. The excitation beam was attenuated so that the ratio of the rates of “stop” to “start” pulses was always less than 2%. The outputs of the time-to-amplitude converter were processed by a multichannel analyzer (Tracor-Northern 1706), and the accumulated data sent to a VAX 11/780 computer for analysis. The instrument response functions were measured by scattering excitation light with a dilute solution of coffee creamer. We typically obtained 255 ps for the FWHM of the instrument function.

Since only one trace can be accumulated at a time, the parallel and perpendicular curves collected separately must be properly normalized with respect to cumulative excitation intensity. Fluctuations in the intensity of the laser made it impossible to do this accurately by simply collecting curves for equal lengths of time, so the scaling was done by using an intensity integrator which monitored a portion of the excitation beam picked off by a beam splitter. The integrator consisted of a photo-
multiplier (1P28), a voltage to frequency converter, and a counting circuit which thus incremented at a rate proportional to the laser intensity. When a preset value in the counter was attained, a signal was sent to the multichannel analyzer which stopped the experiment. It was found by measuring fluorescence from samples that rotated more rapidly than the system response time that the normalization error not compensated for by the integrator was less than ± 1%.  

Spectroscopic measurements were made on dilute unbuffered solutions of eosin and lysozyme with concentrations ranges of 0-30 μM and 0-300 μM, respectively. The pH of the mixtures was measured to be ~5.3. Eosin Y (Eastman Kodak) was purified by thin layer chromatography on a silica plate (60 F-254 Manufacturing Chemists, Inc.) using a solvent mixture consisting of 25:15:30, Ethanol: Chloroform: Ethyl Acetate, respectively, by volume. Lysozyme (3X Crystallized, Dialyzed, and Lyophilized Powder, Sigma Chemical Co.) was used without further purification. The concentrations of the unmixed solutions were determined spectroscopically using published values of the molar extinction coefficients of $\varepsilon(\lambda = 516 \text{ nm}) = 9.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for eosin and $\varepsilon(\lambda = 280 \text{ nm}) = 3.65 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for lysozyme.

Measurements were made on solutions with various concentrations of eosin and lysozyme, generally excess in lysozyme to insure that essentially all the eosin was bound (except for one set of experiments, see below). Using the value of the binding constant given by Baughner et al. of $2.2 \times 10^3 \text{ M}^{-1}$, we calculated that for all experiments with excess lysozyme, at least 96% of the eosin was bound. Most measurements were done with lysozyme and eosin concentrations of $1.2 \times 10^{-4} \text{ M}$ and $3.8 \times 10^{-6} \text{ M}$, respectively (96% bound, calculated). When duplicate measurements were made on solutions with three times that concentration of lysozyme, no differences were observed.

**Data Analysis**

Polarized emission data was analyzed by simultaneously fitting pairs of parallel and perpendicular curves to convolutions of the appropriate combinations of model functions for K(t) and r(t) with parameters varied iteratively to minimize $\chi^2$. The quality of the fits was judged according to how well the statistical tests of $\chi^2$ and the number of runs in the set of generated residuals were satisfied.

The anisotropy data was analyzed by simultaneous fitting of parallel and perpendicular decay curves using the method previously described. In order to obtain the long time behavior of the anisotropy which describes the overall reorientation of the protein, the latter portion (starting at about 5 ns after the initial rise) of the decay curves were first fit assuming a single exponential anisotropy decay law, defined by the parameters r(0+) and $\tau_r$. For these fits, the shift parameter (introduced in convolution to account for the wavelength dependent transit time of the photomultiplier) is fixed at a value determined by magic angle experiments on free or bound eosin. This is done because the time origin must be defined in order to extrapolate back to obtain r(0+). This information is used with the measured r(0) in evaluating the order parameter, given by
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\[ S^2 = \frac{r(t)}{r(0)} e^{+t/\tau_f} = \frac{r(0+)}{r(0)} \quad (1) \]

which gives an indication of what motions are present in addition to the overall reorientation.

The possibility of an nonexponential anisotropy decay was examined by fitting the data over its entire range (including the initial rise) to both single exponential and double exponential anisotropy decay laws. The parameters for the double exponential form for the anisotropy are defined by:

\[ r(t) = r^{(1)}(0)e^{-t/\tau_1} + r^{(2)}(0)e^{-t/\tau_2} \quad (2) \]

where \( \tau_1 > \tau_2 \) by convention. A second model independent parameter \( \tau_e \), the effective correlation time of the short component in the anisotropy decay can be obtained. In terms of \( \tau_1 \) and \( \tau_2 \) obtained from a double exponential fit,

\[ \frac{1}{\tau_e} = \frac{1}{\tau_2} - \frac{1}{\tau_1} \quad (3) \]

In each case, the parameters for \( K(t) \) were fixed at values determined by magic angle experiments (see Table I), and the \( r(t) \) parameters were allowed to vary independently of each other.

**Pure Eosin**

The fluorescence decay of eosin has been extensively investigated. The fluorescence is single exponential with a decay time of 1.100 ± 20 ps in water at 20°C (pH 5.3). There is a small increase in lifetime with increasing temperature. The increase is roughly linear and the change is about 20% from 5°C to 40°C. The reorientation of eosin has also been studied in aqueous solution and the anisotropy decay is well described by a single exponential \( (\tau_r = 154 ± 10 \text{ ps}, \text{H}_2\text{O}, 20°C, \text{pH 5.3}) \). The limiting value of the anisotropy is at the theoretical value within experimental error \( (r(0) = 0.40 ± 0.02) \). For comparison with the curves obtained from the bound eosin, parallel and perpendicular polarized fluorescence decay curves of pure eosin are shown in Figure 1.

**Fluorescence Decay Law of Eosin-Lysozyme Complex, \( K(t) \)**

The fluorescence decay law of eosin bound to lysozyme was first measured in experiments in which the analyzer polarizer was set at the magic angle to the direction of incident polarization. In all cases where visible excitation (514.5 nm) was used, the emission decay law was nonexponential, in contrast to that of unbound eosin. However, the data fit well to double exponential decays; the results of these fits are summarized in Table I. Typical results of fitting are shown in Figure 2.
Figure 1. Simultaneous fitting results for free eosin in water at 20°C. Fitted curves and data points, are shown for parallel and perpendicular fluorescence, using a fixed excited state decay law, $K(t) = \exp(-t/1070 \text{ ps})$. The anisotropy obtained is $r(t) = 0.39\exp(-t/154 \text{ ps})$. The shift parameter was 57.5 ps. Weighted residuals for both curves are shown, the upper and lower plots are for parallel and perpendicular curves, respectively. Statistical parameters obtained for this fit are $\chi^2_r = 1.109, Z = 2.431$; Runs = 453, $Z = -2.5$. For discussion of statistical tests, see reference 16.

Figure 2. Polarized emission curves for eosin ($4 \times 10^{-6}$M) bound to lysozyme ($6 \times 10^{-3}$M) at 5°C. Two sets of residuals are shown, the upper set for a single exponential $r(t)$ and the lower set for a double exponential model $r(t)$. The single exponential fit gives $r(t) = 0.33\exp(-t/9812 \text{ ps}), \chi^2_r = 1.6, Z(\text{runs}) = -4.0$; the double exponential fit gives $r(t) = 0.32\exp(-t/10860 \text{ ps}) + 0.095\exp(-t/62 \text{ ps}), \chi^2_r = 1.25, Z(\text{runs}) = -3.9$. Fit to the long time portions of the curves ($t > 6 \text{ ns}$) gave $r(0+) = 0.314, \tau_r = 11.01 \text{ ns}, \chi^2_r = 1.069, Z(\text{runs}) = -2.2$ (residuals not shown). The fitted curves drawn are for the double exponential $r(t)$. 
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Table I
Magic Angle Data for Eosin/Lysozyme Complex*

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>% A₁</th>
<th>r₁ (ps)</th>
<th>% A₂</th>
<th>r₂ (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>80.3 ± 0.8</td>
<td>2290 ± 13</td>
<td>19.7 ± 0.8</td>
<td>910 ± 70</td>
</tr>
<tr>
<td>34</td>
<td>74.0 ± 0.4</td>
<td>2240 ± 2.2</td>
<td>26.0 ± 0.4</td>
<td>820 ± 30</td>
</tr>
<tr>
<td>40</td>
<td>72 ± 3</td>
<td>2300 ± 96</td>
<td>28 ± 3</td>
<td>940 ± 30</td>
</tr>
<tr>
<td>55</td>
<td>66 ± 2</td>
<td>2200 ± 26</td>
<td>34 ± 2</td>
<td>870 ± 60</td>
</tr>
<tr>
<td>65</td>
<td>62.2 ± 0.6</td>
<td>2160 ± 30</td>
<td>37.8 ± 0.6</td>
<td>800 ± 100</td>
</tr>
</tbody>
</table>

*Error limits are standard deviations from mean of several measurements.

The average value of the fluorescence lifetime, \( <\tau> = (A₁τ₁ + A₂τ₂) \), is about two times larger than the decay time of the free eosin. In contrast to Baugher et al.\(^{24}\) (see also Fisher et al.\(^{39}\)) we find a small increase in the quantum yield of bound vs free eosin. The decay times of the bound eosin are relatively insensitive to temperature (Table I).

Anisotropy of the Complex, \( r(t) \)

The results of the anisotropy analysis for the eosin-lysozyme complex are given in Table II. The most striking result of the fits to the long time portion of the polarized fluorescence curves was that the \( r(0^+) \) value was significantly lower than \( r(0) \) measured for isolated eosin. We believe that this indicates the presence of a rapid

Table II
Anisotropy Data for Bound Eosin*

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Asymptotic Fit</th>
<th>Single Fit</th>
<th>Double Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r(0+)</td>
<td>( \tau_0 ) (ns)</td>
<td>( \tau_1 ) (ns)</td>
</tr>
<tr>
<td>5</td>
<td>0.33</td>
<td>12.4 ± 2.2</td>
<td>0.35 ± 0.4</td>
</tr>
<tr>
<td>34</td>
<td>0.30</td>
<td>4.8 ± 0.2</td>
<td>0.30 ± 0.2</td>
</tr>
<tr>
<td>40</td>
<td>0.31</td>
<td>3.6 ± 0.7</td>
<td>0.29 ± 0.2</td>
</tr>
<tr>
<td>55</td>
<td>0.25</td>
<td>3.4 ± 1.1</td>
<td>0.26 ± 0.3</td>
</tr>
<tr>
<td>65</td>
<td>0.25</td>
<td>2.6 ± 0.8</td>
<td>0.26 ± 0.2</td>
</tr>
</tbody>
</table>

*Error limits are standard deviations from mean of several measurements.
motion of the bound eosin, but the values of the overall reorientation time are also of interest and will be discussed first.

**Diffusive Tumbling of Lysozyme**

The overall reorientational motion of the lysozyme molecule is of interest for several reasons; for example, an accurate description of the reorientation process is needed for analysis of magnetic resonance studies of protein dynamics.\(^4\) The reorientation time of lysozyme has been studied previously by a variety of techniques, including light scattering,\(^31,32\) NMR,\(^33\) and steady state fluorescence,\(^34\) reflecting the absence of a clear method of choice for measuring this quantity. Due to the sensitivity to errors peculiar to each technique, there is a fair amount of variation among reported values. In particular, Dill and Allerhand\(^35\) note the necessity for very accurate C-H bond lengths in the NMR studies and the steady state fluorescence studies suffer from uncertainty in excited state lifetime and form of the fluorescence and anisotropy decays. The \(\tau_r\) extracted by all of these methods might also be influenced by rapid internal motions of the protein or the attached probes. In addition, less sensitive techniques may observe both monomeric and higher aggregate protein motion since relatively concentrated solutions are required. Using the values of the monomer: dimer equilibrium constant given by Banerjee et al.\(^35\) (\(K = 345 \text{ M}^{-1}\)), we conclude that in the solutions of lysozyme used in our study (1.2 \(\times\) 10\(^{-4}\)M) less than 8% of the lysozyme present was in the dimeric form. Concentrations higher by two orders of magnitude were used by some previous workers.\(^31,32\) Consequently, we may have 8% of the eosin bound to lysozyme dimers in our experiments, but the binding constant dictates that it is not possible to avoid this problem and still maintain a condition of excess lysozyme where almost all of the eosin is bound. Finally, since lysozyme is not spherical\(^3\) differing techniques may not detect the same component of the anisotropic reorientation.

A recent survey by Delepierre et al.\(^36\) suggests a mean value of \(\tau_r = 10\pm2\) ns at 34°C in D\(_2\)O, which corresponds (assuming \(\tau_r \propto \eta/T\)) to 8.2\(\pm\)1.6 ns at 34°C in H\(_2\)O. Our results are not in very good agreement either with this suggested mean value, with the light scattering results of 10\(\pm\)0.5 ns at 20°C obtained by two groups,\(^31-32\) or with the value of 21 ns at 5°C obtained by binding fluorescein to lysozyme and measuring steady state fluorescence polarization.\(^34\) In contrast, our results agree well with the NMR measurements of Dill and Allerhand.\(^33\) It is also useful to compare the three calculated characteristic reorientation times \(\tau_a = (6D_{\perp})^{-1}, \tau_b = (5D_{\perp} + D_{||})^{-1},\) and \(\tau_c = (2D_{\perp} + 4D_{||})^{-1}\) of a symmetric prolate ellipsoid with major axes 45 Å x 30 Å x 30 Å, suggested by Artymiuk, et al.\(^3\) as approximating well the shape of lysozyme obtained from crystallographic data. The results of these calculations and the experimental values are illustrated in Figure 3. In general, a weighted combination of these three relaxation times will be observed with the weighting factors determined by the orientation of the eosin transition moments with respect to the symmetry axis of the ellipsoid.\(^37,38\) The three times are quite similar because of the small axial ratio (1.5), and it is unlikely that it will be possible to resolve nonexponential behavior in r(t) due to anisotropic reorientation. We
indeed found no evidence for nonexponentiality on a nanosecond time scale for \( r(t) \). The calculated values (again assuming a \( \tau_r \propto \eta/T \) temperature dependence) are in excellent agreement with our measured values.

Fitting the entire decay curve with a single exponential anisotropy gives a slightly shorter value of \( \tau_r \) than is given by fitting only the asymptotic portions of the measured curves. Fitting \( r(t) \) as a double exponential gives a \( \tau_1 \) (long component) value rather close to the \( r(0^-) \) value as would be expected given the very short decay of the second component in the anisotropy.

**Short Component in the Anisotropy of Bound Eosin**

The data in Table II provide evidence for the existence of a short component in the decay of the fluorescence anisotropy of the bound eosin. Both the single exponential fit to \( r(t) \) and the value of \( r(0^-) \) obtained by extrapolation of the long time behavior of \( r(t) \) back to zero time are significantly less than 0.4. In the case of free eosin, we find that a single exponential fit to \( r(t) \) gives the theoretical initial value of the anisotropy within experimental error\(^{16} \) (\( r(0) = 0.40 \pm 0.02 \)). The existence of a short component in \( r(t) \) is also supported by the double exponential fits to \( r(t) \) shown in Table II. The measured value of \( r(0) = r(1)(0) + r(2)(0) \) is 0.4 within experimental error, and a short component with a decay time of \( \sim 100 \text{ ps} \) is found. Before interpreting our data we must rule out the possibility that the trends we have observed simply result from the presence of free eosin or from the temperature dependence of the magic angle decay parameters. These issues are addressed in the next two sections.

**Does the Short Component in the Anisotropy Arise From Free Eosin?**

In view of the biexponential decay of the magic angle fluorescence from the complex, in which one of the components is quite similar (910 ps @ 5°C) to the decay time of
free eosin (1100 ps @ 5°C), the possibility arises that the short component in the anisotropy of the complex results from free eosin. Since (Table I) the weight of the 900 ps component increases with temperature it might be that the increased amplitude of the short component in the anisotropy simply results from a greater fraction of free eosin at the higher temperature. In this section we attempt to show that our data are not consistent with this idea and that the short component in the anisotropy does indeed reflect motion of the bound eosin.

We begin with the expression for parallel and perpendicular intensities, and the anisotropy for the case where several species with different reorientation functions emit: 19

\[ i_\parallel(t) = \frac{1}{3} \sum_{i=1}^{N} k_i(i)K(i)(t)[1 + 2r(i)(t)] \]  

\[ i_\perp(t) = \frac{1}{3} \sum_{i=1}^{N} k_i(i)K(i)(t)[1 - r(i)(t)] \]

\[ r(t) = \frac{i_\parallel(t) - i_\perp(t)}{i_\parallel(t) + 2i_\perp(t)} \]

where \( K(i)(t) \), \( r(i)(t) \), and \( k_i(i) \) are the fluorescence decay law, anisotropy decay law, and radiative rate constant for emission from species \( (i) \), respectively, and there are a total of \( N \) species. An interesting consequence of the expression for \( r(t) \) is that in a two component system where the shorter lived species has a more rapidly decaying anisotropy, the observed anisotropy may actually increase at longer times, after the population of the rapidly moving species has decayed. This phenomena has been observed by Hudson and coworkers in the anisotropy of probes in membranes. 39

Using expressions (4-6) we have calculated the anisotropy curves to be expected for various cases and compared the results with our experiments. In these calculations we assume that the radiative rates (but not the spectra) of free and bound eosin are identical. In view of the insensitivity of the xanthene dye radiative rate to solvent change 21 this seems a safe assumption.

To facilitate this comparison, the point-by-point anisotropy, \( R(t) \), was computed from some experimental data and the simulated data sets. The point-by-point anisotropy is constructed directly from the measured (convoluted) decay curves:

\[ R(t_i) = \frac{I_\parallel(t_i) - I_\perp(t_i)}{I_\parallel(t_i) + 2I_\perp(t_i)} \]

where \( I_\parallel(t_i) \) and \( I_\perp(t_i) \) are the measured intensities (i.e. number of counts) for the polarized emission curves. These measured intensities are given by convoluting (4, 5) with an instrument response function, \( g(t) \) : 16
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\[ I_{||}(t) = \int_0^t g(t-\tau) i_{||}(\tau) d\tau \]  
(8)

\[ I_{\perp}(t) = \int_0^t g(t-\tau) i_{\perp}(\tau) d\tau \]  
(9)

Since \( I_{||}(t) \) and \( I_{\perp}(t) \) are distorted by convolution,\(^{16}\) \( R(t) \) is distorted, and for example is often nonexponential at short times, even if the true anisotropy decays as a single exponential.\(^{16}\) The features of graphs of \( R(t) \) have been discussed extensively by us,\(^{16}\) and others.\(^{40,41}\)

The experiments are simulated by generating the appropriate combinations of \( r(t) \) and \( K(t) \) for each species, and then combining them to give deconvoluted data for \( i_{||}(t) \) and \( i_{\perp}(t) \). These data are then convoluted with a typical experimental instrument function to obtain the expectation values of the number of counts in each channel of the decays. Noise is simulated by adding to each channel a pseudorandom number chosen from a Gaussian parent distribution with mean and variance given by the expectation value computed for that channel, as is appropriate for counting statistics.\(^{42}\) The \( R(t) \) curves are then computed for pairs of parallel and perpendicular data sets.

Simulations were carried out based on two alternate hypotheses: (1) that the 900 ps component observed in the eosin:lysozyme mixtures is free eosin only, and that the bound eosin undergoes no rapid restricted motion but reorients only with the overall lysozyme tumbling or (2) that the bound eosin exhibits biexponential fluorescence decay (perhaps because of two binding sites, see below) and does have a temperature dependent short component in its anisotropy.

The results of the simulations and the experimental point by point anisotropies \( (R(t)) \) at 65°C are shown in Figure 4. According to hypothesis (1) we have 38% free eosin with a reorientation time of 57.5 ps (obtained by scaling 154 ps at 20°C\(^{48}\) by \( \eta/T \)) and 62% bound eosin with 2.64 ns reorientation time. In hypothesis (2) we use the parameters for Table I and Table II (double fit). Figure 4a shows the hypothesis (1) curve. It is very different in shape from hypothesis (2), Figure 4b, or the actual data (Figure 4c); the latter two decay exponentially at times > 2 ns. The 'lump' in Figure 4a between 2 and 5 ns results from the increasing dominance of the slowly moving bound eosin in the anisotropy. Hypothesis (2) and the real data are superimposable. A similar set of curves was generated using the 5°C data with identical results. Again the hypothesis of free eosin (20% in this case) produces an \( R(t) \) quite distinct from the experimental curve (generated from the data shown in Figure (2) or from the simulated curve assuming only bound eosin with double exponential fluorescence and anisotropy.
As a second check we carried out experiments on a solution (at 5°C) not excess in lysozyme, where we expect significant free eosin. From concentrations determined spectroscopically and the published value of the binding constant,\textsuperscript{24} 12% of the eosin in this solution was calculated to be bound. In order to predict the fluorescence emission under hypothesis (2), the ratio of absorbances of free to bound eosin must be taken into account. At 514.5 nm, this ratio is 1.5. Thus, if 12% of the eosin is bound, we predict that the magic angle fluorescence decay should have the following three components: 91.6% 1.03 ns, 1.6% 0.91 ns, and 6.8% 2.29 ns. Under normal conditions, we would not expect to resolve the difference between the first two components and in fact a double exponential fit to the measured decay gave 7.9% 2.02 ns and 92.1% 0.99 ns with a $\chi^2$ of 1.064, in good agreement with the prediction. However, these values can also be interpreted as 7.9% bound and 92.1% free according to hypothesis (1) so the magic angle data above do not constitute a good test.

The polarized emission curves for this mixture are shown in Figure (5) and the $R(t)$ curve from them in Figure (6c). Although the data points become very scattered after 5 ns we believe that they show an increasing anisotropy at long times. Figure (6b) shows a simulation assuming hypothesis (2) with 8.3% emission from bound eosin and 91.7% from free eosin. Again a curve very similar to experiment is obtained. However, in this case, hypothesis (1) (with 8% emission from bound eosin as calculated from the magic angle weights) produces a similar, but not identical, curve (Figure 6a). It would be difficult to argue strongly, from these data alone, that
the short decay component arises either solely from free eosin or a combination of free and bound eosin. However, in conjunction with the results shown in Figure 4, we believe these curves do give confidence in the value for the binding constant. In Figure 6 when we definitely do have free eosin the predicted (but perhaps non-intuitive) upturn in the anisotropy is indeed observed, but is absent in the experimental R(t) plots (Figure 4) when we predict 100% bound eosin. We therefore conclude that for all the data presented in Table II result from solutions in which essentially all the eosin is bound.

Does the Temperature Dependence of the Order Parameter Arise from Heterogeneity in the Binding Site?

In light of the conclusion of the previous section, the most straightforward explanation for the nonexponential decay of the bound eosin is that there are two binding sites in which the radiationless decay of the eosin differs (by "binding site" we include the possibility of differing conformations in the same site). If this is correct then the relative affinities of the two sites must be temperature dependent since the weight of the short component increases from 20% at 5°C to 38% at 65°C. In this section we attempt to rule out the possibility that the temperature dependence of the weights of the fluorescence decay components causes us to see the observed changes in the anisotropy, without any necessary temperature dependence in the rapid motion.

If we accept that bound eosin does display a rapid component in its anisotropy, then we must have a rapid component for at least one binding site (or conformation).
Figure 6. Point-by-point anisotropies calculated for two alternate hypotheses and experimental data for a mixture of 12% bound and 88% free eosin at 5°C. a) hypothesis (1) (see text) assuming 7.9% emission from bound eosin with $\tau_r = 12.35$ ns, $\tau_K = 2.02$ ns; 92.1% emission from free eosin with $\tau_K = 0.99$ ns, $\tau_r = 246$ ps. b) hypothesis (2) assuming 8.3% emission from bound eosin with double exponential $K(t)$ and $R(t)$ given by parameters in Tables I and II, and 91.7% free eosin with $\tau_K = 1.01$ ns, $\tau_r = 246$ ps. c) $R(t)$ calculated from experimental data in Figure 5. Counting noise (see text) was added to the curves used to generate a) and b).

One possibility that would seriously alter the interpretation of our data is that one site exhibits rapid but temperature independent motion but in the other site the eosin is rigidly held. We have examined the consequences of this suggestion using the same techniques as in the previous section.
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The first observation is that, in order to reproduce the experimental $r(0+)$ values, the long (2 ns) fluorescence component must contain a short anisotropy component. The reason for this is that the procedure for obtaining $r(0+)$ (see experimental section) is not very sensitive to the 900 ps component since the fits begin after most of it has decayed. All simulations therefore were done for this case.

Simulations were carried out for a model in which the 900 ps component had only the overall lysozyme motion, and the 2 ns component had the correct amount of (temperature independent) short anisotropy component (100 ps) to reproduce the observed $r(0+)$ at 5°C. The $r(0+)$ value was then calculated at 65°C using the data of Table I and the overall rotation time from Table II. In the light of the previous paragraph it is perhaps not surprising that the $r(0+)$ value at 65°C is in fact the same as at 5°C, in contrast to the experiments. This result is confirmed by double exponential anisotropy fits to these simulations which give unchanged $r^{11}(0)$ and $r^{12}(0)$ at the two temperatures. These double exponential fits are good, even though the calculated anisotropy does not decay strictly as a sum of two exponentials. We do not feel it possible with the present analysis to rule out a significant difference in the mobility of the two sites, however we can definitely say that the rapid motion in either site must be temperature dependent in order to reproduce our experimental data.

Rapid Motion of the Eosin

Since we have convincingly ruled out contamination of the complex anisotropy decay by free eosin, we feel confident in concluding that there is a fast component in the anisotropy decay of the bound eosin. In view of the free eosin result it seems unlikely that this short component results from electronic relaxation. If there are indeed two binding sites (orientations) of the eosin in order to interpret the data we now make the simplifying assumption that the observed motion is the same in both sites. Since we have shown that rapid motion must occur in at least one site, the most serious problem that this assumption introduces is that the motions we observe arise from behavior averaged over both sites. The binding site of eosin is believed to be the “hydrophobic box” region of the lysozyme. Proton NMR studies of several residues in the hydrophobic box region by Delepierre et al. led to the conclusion that the side chains of the Met 105 and Ile 98 showed considerable angular fluctuations ($\theta_0 = 64° \pm 23°$ for the $C^\beta H_\alpha$ group of Met 105, for example). The hydrophobic box includes approximately residues 17-28 and 95-109. From their crystallographic studies, Artymiuk et al. found a peak in the mean square harmonic amplitude for residues 101 and 102 and larger than usual mean square amplitudes over the whole range 98-109. Thus, it seems likely that there is considerable motion in the hydrophobic box region of lysozyme and we suggest that our data reflects rapid restricted motion of the eosin molecule in its binding site as a result of the amino acid motions.

Lipari and Szabo and Levy and Szabo have suggested that anisotropy results should be discussed in terms of the model independent order parameter $S^2$ and correlation time $\tau_e$. The values of $S^2$ calculated from the data in Table II and the
Figure 7. Order parameter $S^2$ as a function of temperature for eosin-lysozyme complex. Experimental points (O) calculated from the asymptotic fit data in Table II are shown. The solid line is a linear least-squares fit to the data. Dashed lines give predicted temperature dependence calculated from equation (11) either by extrapolation back from 70°C or forward from 0°C.

The definition of $S^2$ in equation (1) are shown in Figure 7. A linear least-squares fit to the data gives $S^2 = 0.86$ at 0°C and $S^2 = 0.61$ at 70°C. This decrease of $S^2$ with increasing temperature indicates that the motion of the bound eosin is less restricted at higher temperatures.

After the data has been expressed in terms of the model independent order parameter, the next step is to explore what reasonable models for the form of the restricted motion are consistent with the observations. That is, given a model of the motion, what are the predicted values of the order parameter across the measured temperature range?

Following Lipari and Szabo\textsuperscript{14} for motion that is azimuthally symmetric about an axis

$$S = \langle P_z (\cos \theta) \rangle$$

where $\theta$ is the angle between $\vec{\mu}$ and the symmetry axis. Several previous workers\textsuperscript{10,13} have interpreted their data in terms of a "cone" model, in which a vector associated with a chromophore (in our case, the transition dipole moment) is assumed to move freely within a cone with fixed semi-angles and symmetry axis fixed with respect to the body fixed frame of the protein. In this case, the relation between $S$ and the cone semi-angle $\theta_0$ has been given by Lipari and Szabo

$$S = \frac{1}{2} \cos \theta_0 (1 + \cos \theta_0)$$

From Figure 7 we find $\theta_0 = 18^\circ$ at 0°C, and $\theta_0 = 32^\circ$ at 70°C. The temperature dependence of this model is generated exclusively by making $\theta_0$ a function of temperature; if this is done empirically, the cone model will always fit the data, so our results cannot constitute a test of this model. However, if an Arrhenius dependence of $\theta_0(T)$ is assumed, we obtain an activation energy of $\sim 500$ cm\textsuperscript{-1}, which seems reasonable.
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However, we can carry the analysis of this model a step further by interpreting the values of the effective cone angles in terms of the structure of the complex. In order to examine the possible structures of the complex, a molecular model of the hydrophobic box region of lysozyme was constructed from space-filling atoms using the coordinates from crystallographic data. The most reasonable structure of the complex inferred from this model has the xanthene plane of the eosin held between the top and bottom of the hydrophobic box. The fit appears sufficiently tight to imply that any motion of the dye is a direct reflection of motions of the residues which surround it, rather than motions of the eosin alone with respect to the walls of the hydrophobic box. It is likely that in addition to this tilting of the ring caused by movement of the walls, there also occurs some sliding or turning of the eosin about a vector normal to the ring plane. Since the transition dipole is along the long axis of the xanthene ring system, these two types of motions can be combined to describe a region within which the transition dipole apparently moves.

Assuming that the extents of these motions are the same, and that the cone model applies, we can translate the measured angles to distances that the residues move using the known length of the eosin dye. If the semi-angle of the cone is $\theta_0$, and the length of the chromophore is $L$, we obtain for the maximum excursion of the ends of the molecule, $X = L \sin \theta$ by applying the law of cosines. The length of the long axis (parallel to the relevant transition dipole moment) is about 14 Å, so the measured cone angles at 0°C and 70°C correspond to maximum excursion distances of 4.3 Å and 7.4 Å, respectively. That is, the extent of motion has increased by about 70% over the measured temperature range. This large percentage increase is not unreasonable in light of molecular dynamics calculations. Levy and coworkers studied the temperature dependence of the motion of an $\alpha$-helical polypeptide over the range 5K to 300 K. They obtained an increase of 30% in the root mean square displacement of the central atoms from 250 K to 300 K. The increase of $\langle \Delta R^2 \rangle$ with temperature became more rapid at higher temperatures so it is not unreasonable to take 40% (assuming a range of 65 instead of 50 degrees) as a lower limit of the predicted increase. Our measured value is probably larger because the groups which define the hydrophobic box of lysozyme, particularly residues 95-109, are not typical $\alpha$-helices, and there is evidence for atypically large motions in these regions.

An alternate model is to assume that the probe moves in a harmonic potential, $V(\theta) = \lambda \theta^2$, and that the probability of finding the probe at $\theta$ is determined by a Boltzmann factor. If the shape of the potential is temperature independent (i.e., $\lambda$ does not depend on temperature) the variation of $S$ with temperature arises solely from the increase in the amplitude of the motion as $k_B T$ increases. The expression for $S$ from this model is

$$S(T) = \frac{\int P_2(\cos \theta) \exp(-V(\theta)/k_B T) \sin \theta d\theta}{\int \exp(-V(\theta)/k_B T) \sin \theta d\theta}$$

(12)
For a moderately restricted angular range, the upper limits of integration can be set to infinity with little error. In this case the result is

\[
S(T) = \frac{3F\left(\frac{3\sqrt{a}}{2}\right) - F\left(\frac{\sqrt{a}}{2}\right)}{8F\left(\frac{\sqrt{a}}{2}\right)}
\]

(13)

where \( a = \frac{k_B T}{\lambda} \) and \( F(x) \) is Dawson's integral.\(^4\) Using the measured value of \( S^2 \) at a particular temperature we can solve equation (13) for \( \lambda \), and predict \( S^2(T) \). Since \( \lambda \) is the sole parameter of the model. From the least-squares fit to the data and numerical solution of equation (13), we obtain \( \lambda = 1.16 \text{ cm}^{-1}\text{deg}^{-2} \) at 0\(^\circ\)C and \( \lambda = 0.426 \text{ cm}^{-1}\text{deg}^{-2} \) at 70\(^\circ\)C. The temperature dependence of \( S^2 \) predicted from either of these values is shown in Figure 7, and evidently is too weak to explain our results.

Levy et al.\(^9\) in a molecular dynamics simulation of an \( \alpha \)-helical polypeptide, concluded that a harmonic approximation significantly underestimated the amplitudes of displacements. Fraunfelder and coworkers\(^2\) have analyzed crystallographic data in terms of anharmonic potential functions. The possibility of using an effective anharmonic potential to explain the observed temperature dependence of the order parameter can be explored by numerically solving equation (12) using a temperature-independent potential of the form \( V(\theta) = \lambda \theta^4 \), where the parameter \( \mu \) has been introduced for a more general form. For a particular fixed value of \( \mu \), it should be possible to find the value of \( \lambda \) which gives the value of \( S^2 \) at a particular temperature (e.g. 5\(^\circ\)C). Using equa. (12), one could then determine the predicted temperature dependence of \( S^2 \) for the fixed values of \( \lambda \) and \( \mu \). The temperature dependence of \( S^2 \) will require a \( \mu < 2 \) (for fixed \( \lambda \)). We are proceeding with an analysis along these lines and will report the results in a future publication.

Timescale of the Rapid Motion

The value of \( \tau_2 \) is essentially the same as \( \tau_0 \) in our case because \( \tau_2 \) is roughly two orders of magnitude less than \( \tau_r \). Our measurements definitely confirm the existence of the short component, but the measured values of \( \tau_2 \) are subject to rather large uncertainty. With the present time resolution we do not feel it is meaningful to discuss trends in \( \tau_2 \) with temperature. We also note that while evaluation of \( S^2 \) does not assume that the rapid anisotropy decay is exponential, the fitting procedure which gives \( \tau_2 \) does. It seems unlikely that the initial anisotropy decay is purely exponential and experiments with higher time resolution are clearly necessary to address this point. We are currently planning such experiments using the anisotropic absorption or polarization spectroscopy technique.\(^4\)

Comparison with HSA

A similar set of experiments was carried out on human serum albumin (HSA) over the temperature range 5\(^\circ\)C-40\(^\circ\)C. The measurements were carried out at a pH of 6
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where the protein is in the so-called normal form. The excited state decay (isotropic decay) was measured and found to fit well to a sum of two exponentials whose parameters changed slightly with temperature. The latter part of the curves were simultaneously fit to obtain the long time behavior of r(t).

The overall orientation times thus obtained agreed well with the Stokes-Einstein law (we obtained $\tau_r = 52$ ns at $5^\circ$C), but the r(0+) values obtained were not dependent on temperature over this range, in contrast to the eosin-lysozyme complex (we obtained r(0+) = 0.25±0.014). Since the appropriate value of r(0) for tryptophan is not known, it is not possible to compute $S^2$ for the tryptophyl residue in HSA, but the constant value of r(0+) indicates that the form of the potential does not change over this temperature range. Two physical models could explain this observation: either the tryptophan is rigidly held in the protein, or it is undergoing a restricted motion with an angular range that does not depend on temperature.

Concluding Remarks

Our measurements of the fluorescence anisotropy decay of eosin bound to lysozyme have provided a refined value for the overall rotation time of lysozyme. The measurements are at significantly lower concentration than previous studies, and protein aggregation can be safely neglected. Our measurements show a rapid restricted motion of the eosin molecule on the time scale of $\sim 100$ ps. The order parameter associated with this motion decreases with increasing temperature, indicating that the motion of the eosin is less hindered as temperature increases. It is not possible to interpret our results in terms of a temperature-independent harmonic potential. Further work is required to decide if the appropriate description involves a temperature-dependent harmonic potential or a temperature independent anharmonic potential (or a combination of the two). The eosin molecule is believed to bind in the hydrophobic box region of lysozyme and our measurements provide direct time resolved confirmation of the rapid motion of groups in this region inferred from crystallographic studies. NMR studies also find considerable motion of aliphatic residues in the hydrophobic box. It will be fascinating to extend our studies to shorter times to reveal more detail of the rapid motion.

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References and Footnotes


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