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Rotations of tryptophan residues in proteins

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A classic study of the application of dynamic fluorescence depolarization methods to the investigation of protein internal rotations was reported some years ago by Munro *et al.* (1979). The relatively large width of the excitation pulse used in those studies limited the time resolution to about 0.1 nanoseconds. Sommer *et al.* (1985) later measured the anisotropy decay of the single tyrosine residue in lima bean trypsin inhibitor using a femtosecond laser and a streak camera. More recently, Harris *et al.* (1986) have utilized time-correlated single photon counting with the narrow pulses obtained from a mode-locked laser to determine the rotational correlation times of the single tryptophan residue in mutants of T4 lysozyme. On the other hand, the use of differential phase and modulation ratio fluorometry to measure rotational properties of intrinsic fluorophores in proteins has been well established in our laboratory (Alcala *et al.*, 1985; Gratton *et al.*, 1985; Jameson *et al.*, 1986; Prendergast *et al.*, 1986). The high resolution, precision and selectivity of our apparatus enabled us to experimentally assess the theoretical findings of Karplus and co-workers (McCammon *et al.*, 1977; Ichiye & Karplus, 1983) on the motions of tryptophan and tyrosine residues of some selected proteins, in particular lysozyme and bovine pancreatic trypsin inhibitor (BPTI). The molecular dynamics calculations showed that rotational motions of those residues can occur in 1–20 ps with an amplitude of several tens of degrees. This time range can be directly tested using the high-modulation frequencies now available in our pulsed-laser multifrequency phase fluorometer (Alcala *et al.*, 1985).

Materials and methods

Lysozyme was from Boehringer-Mannheim and BPTI from Mobay Pharmaceuticals. Glycerol (gold-label), and *p*-terphenyl (gold-label) were purchased from Aldrich. Glycerol viscosities as a function of temperature were taken

from Segur (1953). All protein preparations were buffered in 50 mM-sodium phosphate, pH 7.0. Lysozyme was chromatographed on Sephadex G-75 to remove higher aggregates. BPTI was determined to be pure by examination of the fluorescence emission spectrum which was almost superimposable on that of pure tyrosine. Excitation wavelength and emission filter (in parentheses) were as follows: 300 nm (Corning 0–52) for lysozyme and 287 nm (Triton X-100) for BPTI. Lifetime measurements were performed with the multifrequency phase fluorometer described by Alcala *et al.* (1985). This system uses a mode-locked argon-ion laser and a synchronously pumped dye laser (Spectra-Physics). U.v. radiation in the 285–310 nm region is obtained by doubling the output of the dye laser by an angle-tuned frequency doubler (Spectra-Physics). The modulation frequency set employed ranged from about 10 to 440 MHz. All lifetime measurements were obtained using *p*-terphenyl in cyclohexane or methanol in the reference cell and with a polarizer set at 35° with respect to the vertical direction in the excitation beam. Differential phase and modulation ratio measurements were collected in the range used for lifetime data. Data analysis was performed using a non-linear least-squares routine. For the lifetime analysis a sum of exponentials was used. The following expression was used for the rotational analysis:

$$r_i(t) = r_{0i} (\sum_j g_{ij} e^{-t/R_{ij}}) \quad (1)$$

$$I_\beta(t) = \sum_i \{ [1 + \beta r_i(t)] f_i e^{t/\tau_i} \}$$

where $\beta = 2$ for the vertical component and $\beta = -1$ for the horizontal component of emission (excitation is vertical). $r_i(t)$ describes the anisotropy decay, $I_\beta(t)$ the intensity decay, g_{ij} represent the pre-exponential factors for the anisotropy decay and R_{ij} the corresponding rotational correlation times. f_i is the pre-exponential factor and τ_i the corresponding lifetime of the intensity decay part. The above equations are transformed to the frequency domain for the fit of the differential phase and modulation ratio data. The approximate amplitude of the internal rotations was calculated as in Munro *et al.* (1979) on the wobbling-in-cone model using the expression $\Phi_{\max} = \cos^{-1} \{ \frac{1}{2} [1 + 8(r_\infty/r_0)^{1/2}]^{1/2} - 1 \}$ where r_0 is the time zero anisotropy and r_∞ is the value of the anisotropy when the internal rotations have equilibrated.

Abbreviation used: BPTI, bovine pancreatic trypsin inhibitor.

Fig. 1. Differential phase and modulation ratio for tryptophan in buffer at pH 6

Excitation at 300 nm; emission seen through a Corning 0-52 filter. The best fit gives a rotational correlation time of 81 ps (a). The other line (b) is a simulated curve for a 10 ps rotational correlation time.

Limit of resolution of rotational rate measurements

The accuracy of the differential phase determination in the measurements reported here is of the order of 0.1° , and 0.001 for the modulation ratio. Fig. 1 shows a measurement of the rotation of tryptophan in buffer at pH 7. The solid line is the fit obtained using a rotational correlation time of 81 ps. The other line in the Figure corresponds to a hypothetical rotation of 10 ps that should be detectable in our experiments. If more than one rotational motion is present, for example tumbling of the protein molecule and internal rotation of the tryptophan residue, and if the two motions have very different rates (more than a factor of 10), it is possible to measure them accurately. In the case of restricted rotations, the measurement of the rotational rate is more difficult. In Fig. 2(a) we report a simulation for a trypto-

Fig. 2. Effect of viscosity on a simulated differential phase experiment assuming a restricted internal rotation of 100 ps in a cone of approximately 40° and an overall protein rotation of 5 ns (a), 50 ns (b) and 250 ns (c)

In this simulation the viscosity affects only the overall rotation.

phan residue rotating in a cone of approximately 40° aperture with a rotational correlation time of 100 ps in a protein of 15 000 M_r . The band at high frequency corresponds to the internal rotation. However, if the rotation is even faster, the band can be out of our measurable frequency range which is from 1 to 500 MHz. The only visible result will be an apparently lower value of the time-zero anisotropy. If the rotational rate of the protein is decreased by increasing the external viscosity, the internal rotation should now be more evident, since the overall protein motion does not contribute to the depolarization (Fig. 2b). In the limit in which the tumbling of the molecule as a whole is blocked, only the internal rotation should remain (Fig. 2c). We have utilized this approach to investigate the internal rotations of tryptophan and tryosine residues in proteins.

Analysis of the anisotropy decay in the presence of multiple lifetime components

Using three different models, we have analysed intensity and anisotropy decay results obtained using differential phase and modulation fluorometry. The need for the use of different models arises from the multi-exponential character of the intensity decay in proteins. Depending on how the lifetime components are associated with different molecular species, different equations can be obtained. The equations describing the decays of the vertically polarized (I_v) and horizontally polarized (I_h) emissions contain the products of exponential terms of the intensity decay and of the rotational diffusion relaxation. If there is only a single lifetime component, it cancels out of the anisotropy expression. If a mixture of emitting species with different lifetimes and rotational rates is present, I_v and I_h contain the sum of several terms (eqn. 1) and the intensity part does not cancel. Moreover, a lifetime component can be interpreted erroneously as a rotational component. In view of the complexity of the general anisotropy expression, we used the simplified assumption that a maximum of two distinct species are present.

(1) The first model considers a single rotating species capable of internal mobility. This is essentially the model which results when the Perrin equation is applied to steady-state measurements ($i = 1$ and $j = 2$ in eqn. 1).

(2) The second model considers two species to be associated with the two lifetime components each having a single rotational motion ($i = 2$ and $j = 1$ in eqn. 1).

(3) The third model is an extension of the second and assigns two rotational motions to each molecular species ($i = 2$ and $j = 2$ in eqn. 1). This latter model contains several parameters and, to be tested adequately, requires the high accuracy in phase and modulation determination that we have in our instrument.

We have found in studying a large number of protein systems that the third model provides better fits of the data than do the other two. The lifetime measurements have indicated that continuous lifetime distributions better describe the observed decay than the sum of several discrete exponential terms. The protein heterogeneity revealed by this analysis is likely to be reflected also in the rotational mobility of the tryptophan residues(s). In our view, a protein can have a large number of different conformations for each of which the tryptophan (or tyrosine, or other) lifetime value and rotational mobility may differ. The use of only two distinct species in our analysis thus constitutes more or less an approximation to such a continuous distribution, but conveys some insight of how to treat the anisotropy data in the presence of multiple lifetime components.

Lysozyme

Fig. 3 shows the analysis of the differential phase and modulation data for lysozyme in 50 mM-phosphate buffer at

Fig. 3. Differential phase and modulation ratio measurements for lysozyme at 20°C

The best fit (solid lines) was obtained using model (3). The rotational correlation time of the whole molecule was about 4.5 ns and the internal correlation time was about 1 ns.

25°C. The value of the reduced χ^2 using model (1) shows that the fit is relatively good; the rotation of the entire protein corresponds to the longer rotational time and the shorter rotational time should correspond to the internal rotation of the tryptophan residues. However, the value of the time-zero anisotropy obtained from the fit is well below the expected value of 0.31 for tryptophan (excitation at 300 nm). A very fast rotation which is completely out of our measurement range will reduce the value of r_0 . Higher modulation frequencies should be used to test directly for this hypothetical rotation. The analysis using model (2) gave a slightly better fit as judged by the lower value of the χ^2 . In this case also, the value of the time-zero anisotropy was lower than 0.31. Model (3), containing more parameters, gave an even better fit and a value for the time-zero anisotropy close to the theoretical value. On studying the differential phase and modulation ratios over a large range of temperature and viscosity, model (1) always gave a lower value of r_0 except at very low temperature and high viscosity, in which regime only one rotation was detectable. Model (2) also suffered from the same problem, although r_0 was always closer to the expected value. Only model (3) consistently gave the correct value of r_0 . The crucial aspect of our measurements is that we never observed a fast (i.e. faster than 100 ps) rotational component. This hypothetical rotational motion should have moved in our frequency window on increasing the viscosity or decreasing temperature. One possibility is that the fast rotation was dependent of temperature and external viscosity. This is clearly not the case since, at low temperature, we can account for all the anisotropy of the indole ring assuming a single rotation. In Fig. 4 we show the dependence of the two measured rotational correlation times on η/T . One motion can be identified with the rotation of the entire protein since it has a value at 20°C in aqueous solution of about 5 ns. As the temperature was decreased and the viscosity increased, the rotational correlation time increased with an exponent of one in the log-log plot of Fig. 4, in accordance with the Stokes-Einstein relation. The second rotation corresponded at 20°C in aqueous solution to a rotational correlation time of about 1 ns, which is probably a result of the faster internal motions of the tryptophan residues. This motion did not

Fig. 4. Analysis of the rotational motions of the tryptophan residues in lysozyme as a function of η/T

Two rotations are distinguishable: rotation of the entire molecule ($\times$); internal rotation (O). The solid line corresponds to the range of rotational correlation times predicted by molecular dynamics calculations.

follow the Stokes-Einstein relation since the exponent in Fig. 4 was about 0.5.

BPTI

Fig. 5 shows the rotational behaviour of the tyrosine residues of BPTI in 50 mM-Tris buffer, pH 7.0 at 20°C. In the analysis, we obtained a single species with a rotation corresponding to the entire molecule (3.3 ns) and a faster component of 0.66 ns. We have also determined the effect of static quenching on the rotations using 0.1 M-phosphate buffer. Again considering a single species, we observed the global rotation of the molecule and an internal rotation of about 0.84 ns, slightly longer than for the unquenched case.

Fig. 5. Differential phase and modulation ratio measurements for BPTI

The upper and lower curves correspond to the molecular dynamics results.

In this case also, we failed to observe the fast motions predicted by the molecular dynamics simulations (Fig. 5).

Conclusions

The measurement of the rotational correlation times of tryptophan residues in lysozyme and tyrosine in BPTI showed no evidence of the fast rotations of relatively high amplitude predicted by molecular dynamics calculations. In contradistinction, two rotational motions were observed which could be identified with the rotation of the entire protein and with the internal rotations of the tryptophan residues. Our approach differs from more usual analyses of the anisotropy decay. We introduced the lifetime heterogeneity observed for many single and multiple tryptophan proteins as an essential ingredient in the anisotropy analysis. We propose that the physical origin of lifetime heterogeneity is also reflected in the rotational motions of the tryptophan. Consequently, a distribution of rotational rates must be considered. In this work we could satisfactorily approximate this distribution of rates using only two rate components. Using this approach, we recovered the correct value for the time-zero anisotropy. On assuming a single species (lifetime homogeneity) on the other hand, a very fast rotational component is predicted to be present. Since we

have not observed such a fast motion, this hypothetical component would have to have a rotational correlation time shorter than 10 ps, and also disappear, without shifting to longer times, as temperature is decreased and solution viscosity increased.

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Phase and modulation optical spectroscopic methods for determining triplet lifetimes and slow rotational diffusion coefficients

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Several time-resolved optical methods are available for measuring the slow rotational diffusion of integral membrane proteins to which an extrinsic probe has been attached. With the exception of the recently described transient fluorescence anisotropy method, in which a non-fluorescent probe is photoactivated to a transient (μ s–ms) fluorescent state (Blatt & Jovin, 1986), the other methods all exploit the optical properties of so-called triplet probes and use as the basis for detection one of the following:

- optical density changes due to either ground-state depletion or triplet-state population (Cherry, 1978);
- delayed photoluminescence, either phosphorescence or delayed fluorescence (Austin *et al.*, 1979; Garland & Moore, 1979; Greinert *et al.*, 1979);
- loss of prompt fluorescence due to ground-state depletion (Johnson & Garland, 1981, 1982).

In many potentially important biological applications these methods are unsatisfactory because of inadequate sensitivity (e.g. optical density measurements) or poor time-resolution due to artefacts accompanying and following the extremely intense photoselection flash (e.g. photoemission methods). Furthermore, the instrumentation does not lend itself readily to convenient application through a microscope to films or single cell surfaces.

In an attempt to overcome these limitations, we have begun to explore the use of multifrequency phase and modulation methods for measuring triplet probe lifetimes and anisotropies. The theory underlying such methods was developed over 50 years ago (Duschinsky, 1933), but its wider application and instrumental realization has required many technical and theoretical advances such as cross-correlation circuits, lasers, electro-optic modulators and

multifrequency data analysis. Descriptions of current multifrequency phase and modulation fluorometers have been given by Gratton *et al.* (1984) and by Lakowicz & Maliwal (1985). Such instruments are designed to operate in the 1–200 MHz frequency range, or higher, and use analogue cross-correlation circuits (Spencer & Weber, 1969) to generate low-frequency signals that can be analysed for phase and modulation. A different approach can be used for working at the lower frequencies (0.01–50 KHz) appropriate for photoexcited states in the microsecond to millisecond time range. Fig. 1 summarizes the construction of the apparatus that we have developed; the most important parts are an acousto-optically modulated argon-ion laser and signal collection directly into a fast digital averaging transient recorder. Analyses of phase angles and modulation are made on the stored and averaged waveforms, rather than with a lock-in amplifier.

Triplet-probes suitable for delayed photoluminescence measurements, such as eosin and erythrosin, suffer from large prompt fluorescence signals which in pulse methods must be time-resolved from the delayed signals. Photomultiplier gating is needed. In phase and modulation methods, the component arising from prompt fluorescence can be resolved and discarded in the data analysis, but its presence greatly diminishes the phase-retardation and demodulation that would otherwise arise from the delayed luminescence signals (Garland & Birmingham, 1986a). The prompt fluorescence component can be separated from the delayed luminescence by superimposing a 0–100% square wave modulation at 1 MHz or so on top of the sinusoidal laser beam modulation already running at 0.01–50 KHz. The prompt fluorescence lifetime is so short (~ 1 ns) that the dark periods occurring at 1 MHz are free of prompt fluorescence, and can be used to construct the signal waveform arising solely from delayed luminescence modulated in the 0.01–50 KHz range. In effect, both pulse and phase methods are combined to exploit the desirable characteristics of each. Further enhancement of the phase and modulation method will include its application to fluorescence depletion (John-

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