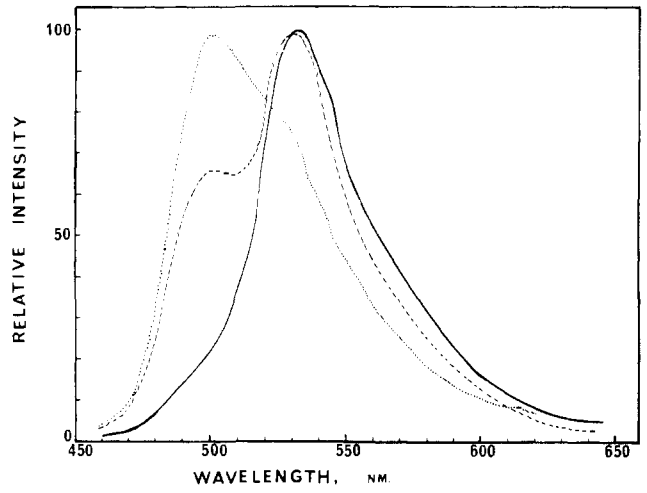

Figure 2. Variation in the fluorescence λ_{max} of bilirubin at 77°K in EPA (ether-isopentane-ethyl alcohol 5:5:2) solution containing varying concentrations of cetyltrimethylammonium bromide: 2.0 mg bilirubin in 25 ml of EPA with no added CATB (——); with 0.6 mg of added CATB (- - -); and 0.05 mg of bilirubin in 25 ml of EPA with 11.6 mg of added CATB (···). Excitation wavelength and bandpass were 390 and 10 nm, respectively.

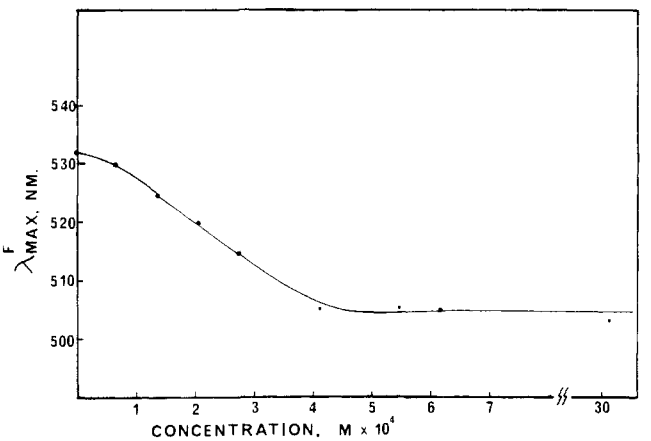

Figure 3. Plot of the fluorescence λ_{max} of bilirubin vs. various concentrations of cetyltrimethylammonium bromide in EPA solutions at 77°K.

The structure of the micelle is as yet uncertain and whether bilirubin is suspended as a monomer is unknown.

It may be noted in Figures 1 and 2 that **1** in aqueous detergent fluoresces at λ_{max} 525 nm; whereas, in EPA-detergent, the λ_{max} is 505. We attribute these differences to differences in conformation (and ionization?) of **1** induced by their different micellar environments. Thus, we propose that, in water, the ionic part of CATB is on the outside and the lipophilic hydrocarbon part of CATB is on the inside of the micelle and bilirubin resides in a strongly lipophilic environment. On the other hand, in EPA, we propose that the relative polarity of the micelle is reversed with the lipophilic surface on the outside and the polar ionic surface on the inside. In the latter, **1** is exposed to a strongly ionic, hydrophilic environment; whereas, in the former it is exposed to a nonionic, lipophilic environment; hence, differences in conformation of **1** are to be expected.

In light of the foregoing, one might therefore expect bilirubin in a lipophilic environment such as EPA alone to exhibit similar fluorescence behavior as in aqueous detergent. Indeed, such is the case as may be noted from a comparison of Figures 1 and 2. Further evidence supporting the notion of similar conformation(s) of **1** in similar environments comes from the observation that **1** also fluoresces at λ_{max} 525 nm in (lipophilic) chloroform solvent at room temperature.

Whether the structure of **1** in our lipophilic environments is akin to that internally hydrogen bonded structure described by Kuenzle et al.[13] cannot be ascertained from our data. We speculate that it might be an important contributor. Furthermore, although little is known of the structure of **1** bound to albumin,[14] it too fluoresces with λ_{max} near 525 nm[3,4] which is suggestive of similar conformation(s) for **1** here as in water-CATB, EPA, and chloroform without albumin.

In contrast to the fluorescence behavior of **1** in nonpolar solvents, when a solvent with a high dielectric constant, acetonitrile, is employed, the fluorescence λ_{max} shifts to ~505 nm with a broad shoulder on the long wavelength side. This behavior is in keeping with that found for **1** in EPA with high concentrations of CATB. However, the fluorescence λ_{max} ≃530 nm (with shoulder at λ_{max} ≃505 nm) for **1** in EPA + sodium methoxide is not entirely expected from the preceding arguments and doubtless reflects an ionization phenomenon (amide as well as carboxyl?) in addition to possible conformational changes.

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Depolarized Light Scattering and Carbon Nuclear Resonance Measurements of the Isotropic Rotational Correlation Time of Muscle Calcium Binding Protein

Sir:

Molecular reorientation is described by the Debye model as random steps of small angular displacements. For macromolecules, the rotational diffusion coefficient, D, can be calculated hydrodynamically from the Stokes-Einstein equation.[1] Rotational Debye motion causes fluctuations in the laboratory reference frame polarizability, if a molecule is optically anisotropic. Depolarized light scattering mea-

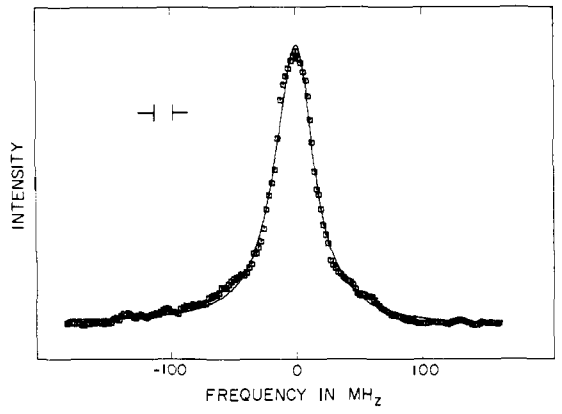

Figure 1. Depolarized Rayleigh spectra of MCBP. The □ are digitized data points and the line is the best fit single Lorentzian. The total number of photon counts/sec was ~500 and a 1-sec time constant was used on the photon counter.

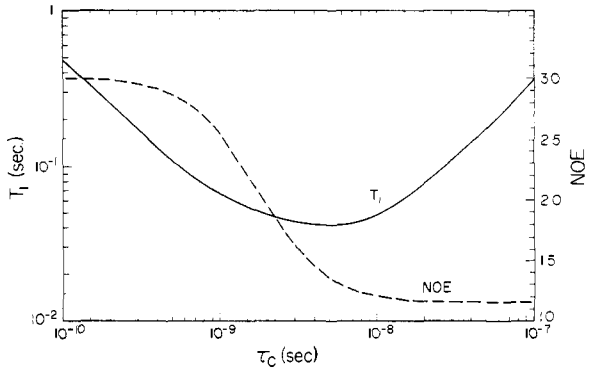

Figure 2. Calculated dipolar T_1 and NOE dependence on τ_c for a carbon-13 nucleus relaxed by a single hydrogen 1.09 Å away in a magnetic field of 23.5 kG, following ref 4b.

sures a rotational correlation time, τ_c, which is an ensemble average of the autocorrelation function of the yz component of the laboratory frame polarizability,[2] α_{yz}

$$\langle \alpha_{yz}(t)\, \alpha_{yz}(0) \rangle \propto e^{-t/\tau_c} \quad (1)$$

Nuclear relaxation parameters, in the case of a carbon-13 nucleus separated from a proton by a fixed distance r, are functions of the correlation time of the dipolar vector between the nuclei. The longitudinal relaxation time (T_1) and nuclear Overhauser enhancement (NOE) can be described in terms of the transition probabilities, W_i, of a two-spin system[3]

$$T_1 = (W_0 + 2W_{1I} + W_2)^{-1} \quad (2)$$

$$\text{NOE} = 1 + \frac{\gamma_H}{\gamma_C}(W_2 - W_0)T_1 \quad (3)$$

The W_i can be calculated from the spectral densities, which are the Fourier transform of a motion dependent autocorrelation function,[4] $G(t)$, which takes a simple form in a molecule undergoing isotropic rotational diffusion

$$G(t) \propto e^{-|t|/\tau_c} \quad (4)$$

τ_c is equivalent to the correlation time of eq 1 and is related to the rotational diffusion coefficient, $\tau_c = (6D)^{-1}$.

Muscle calcium binding protein from carp (MCBP) was isolated by the method of Pechere.[5] This protein has a molecular weight of 12,000 and an unusual amino acid composition with 20% alanine and 10% phenylalanine; the X-ray crystal structure shows that it is a prolate ellipsoid with the approximate dimensions of 30 by 36 Å.

If a protein is undergoing isotropic rotational diffusion it follows from eq (1) that the depolarized light scattering spectral density at frequency shift ω is given by[2]

$$I_{VH}(\omega) = \frac{A\rho}{15\pi}\beta^2\frac{6D}{\omega^2 + (6D)^2} \quad (5)$$

Where A is a constant, ρ is the number density of protein, and β is the optical anisotropy. The line width contains a component which depends on translational diffusion; however, this contribution is negligible for small proteins.

The two techniques that are commonly used in light scattering are light beating and Fabry-Perot interferometry.[7] The latter is most suitable for proteins with a line width greater than 1 MHz. The interferometry apparatus has been described elsewhere.[2] Modifications necessary because of the relatively weak signals and narrow line widths include spherical mirrors with a fixed free spectral range of 750 MHz (providing a stable system with an instrumental half-width of 6-7 MHz) and a 100 Å bandpass filter (removing unwanted Raman and fluorescent light). As can be seen from Figure 1, the spectrum from a 1% solution of MCBP fits well to one Lorentzian. The RMS error in amplitude was 2.5%. The instrumental line shape was deconvoluted from the spectrum[2] and τ_c was found to be 12 ± 1 nsec at 21°. Dubin et al. have previously measured τ_c for hen egg white lysozyme by this method.[8]

If the dominant nuclear relaxation mechanism is dipolar, then the correlation time dependence of eq 2 and 3 is represented in Figure 2. For a slowly reorienting system, such as a macromolecule, T_1 as a function of τ_c passes through a minimum, which makes the unambiguous assignment of a correlation time to a relaxation time impossible. An unique correlation time can be obtained with an additional measurement. The NOE of a resonance determines which side of the minimum the T_1 value represents, since NOE is a sensitive function of τ_c in the region 0.4-10 nsec. It is also possible to use measurements of T_1 at two (or more) field strengths, since eq 2 and 3 are dependent on the resonance frequencies; however, this requires the use of more than one spectrometer. A previous assignment of τ_c to a protein based on the α carbon T_1 used estimates of the overlapping line width to resolve the ambiguity.[9]

The α carbon of an amino acid has one attached hydrogen and because NOE = 3.0 in solution the relaxation mechanism of this carbon is dipolar. The α carbon backbone of a native protein structure is sterically restricted and it may be a reasonable approximation to treat these carbons as if no internal motions are present and their effective correlation time is the same as that of the entire protein. Measurements of nuclear relaxation parameters of α carbon resonances of proteins are necessarily approximate because individual signals cannot be resolved and the behavior of a spectral region must be evaluated; however, this does represent a chemically and structurally homogeneous group.

Inversion-recovery[10] measurements of T_1 of the α carbon region of MCBP indicate a population which relaxes with an apparent single exponential time constant of 50-60 msec. The NOE was measured by the gated decoupler technique[11] and a value of 1.2 ± 0.1 was found. These measurements were made on a 16 mM D_2O solution at 22° in a magnetic field of 23.5 kG.[12] Using the data in Figure 2 the T_1 represents a τ_c of 1.2-2.0 nsec or 11-14 nsec while the NOE reflects a correlation time slower than 10 nsec. The overall reorientation time of the protein can be assigned unambiguously to the value of 11-14 nsec using only the nuclear resonance measurements.

The determinations of the rotational correlation time by nuclear resonance and depolarized light scattering are the same within experimental error and yield a value of about

12 nsec. The finding that the relaxation parameters for the α carbons of this protein are the same as could be calculated from the overall tumbling time implies that these carbons are an integral part of a rigid body, and their motion is nearly isotropic. A hydrodynamic radius of about 22 Å is calculated from the measured value of τ_c and the Stokes–Einstein equation. This radius is about 5 Å larger than the average crystallographic radius, indicating that a significant amount of water is firmly bound to the protein. Several other methods have been used to determine rotational correlation times of proteins including electric[13] and flow birefringence,[14] fluorescence depolarization,[15] dielectric dispersion,[16] and electron paramagnetic resonance.[17] These techniques typically require either the chemical modification of the protein to attach a probe or a severe external perturbation of the sample, limitations not present in the depolarized light scattering and nuclear resonance measurements.

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