Triplet State of Tryptophan in Proteins. 2. Differentiation between Tryptophan Residues 62 and 108 in Lysozyme†

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ABSTRACT: We have used optically detected magnetic resonance (ODMR) to characterize the degree of solvent availability of the tryptophan residues in lysozyme that are likely to be responsible for the observed phosphorescence. From the phosphorescence spectra, ODMR zero-field splittings (zfs), and ODMR line widths, we concur with the X-ray structure [Blake, C. C., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, Ser. B 167*, 365–377] that Trp-62 behaves as an exposed residue and Trp-108 is buried. In addition, we present evidence that ODMR can be used in conjunction with conventional phosphorescence to evaluate the degree of order in the microenvironments of tryptophan in a protein containing several tryptophans. By the specific modification of residues Trp-62 and Trp-108, we have identified those portions of the ODMR lines in the native enzyme that are due to those specific residues. Barring major enzyme conformational changes in the vicinity of *unmodified* tryptophan residues when Trp-62 or Trp-108 are selectively modified, we find that Trp-108 dominates both the phosphorescence and the ODMR signals in native lysozyme. The results are discussed in view of previous fluorescence findings.

There is currently no single technique that will completely ascertain the three-dimensional *solution* conformation of a protein, especially since the protein may experience substantial structural fluctuations, by sampling a range of conformations, each existing for no more than 1 ns, for example (Lakowicz & Weber, 1973a,b; Grinvald & Steinberg, 1976). X-ray crystallography, on the other hand, has successfully determined the structure of several proteins in the crystalline state. Unfortunately, the correlation between crystalline and solution conformation is not straightforward.

There are a number of methods that provide information about the microenvironment of specific residues in the protein. In particular, convincing statements about the environment of the tryptophan residues in aqueous lysozyme have been made based on NMR (Glickson et al., 1971) and fluorescence spectroscopy. For example, Konev (1967) characterized red shifts in tryptophan fluorescence as being associated with a residue exposed to polar solvent and, conversely, blue shifts indicated buried residues. Solvent perturbation of protein fluorescence has been used to indicate the presence of charged groups near tryptophyl side chains (Lehrer, 1971). Several other investigators have analyzed lysozyme fluorescence spectra using spectral parameters—band width, wavelength, quenching, relative quantum yields, lifetimes, and polarization—to examine spontaneous and induced conformational changes [see, for example, Grinvald & Steinberg (1976), McGuire & Feldman (1975), and Burstein et al. (1973)].

Since the tryptophan sites in lysozyme are known from X-ray crystallography (Blake et al., 1967) and since these authors suggest that, owing to the large amount of water of crystallization, the crystal structure of lysozyme is *probably* essentially the same as that of aqueous lysozyme, it serves as a model protein for examining site-specific interactions. In order to analyze and interpret unambiguously the fluorescence spectra in a protein that contains more than one tryptophan, the luminescence of tryptophan in lysozyme must be unraveled to disclose those residues responsible for the composite protein emission. In this context, a controversy has developed over precisely what the relative contribution of each aromatic residue is to lysozyme fluorescence.

By comparing the fluorescence quantum yields of native lysozyme and lysozyme derivatives in which Trp-62 and Trp-108 are rendered nonfluorescent, Imoto et al. (1972) concluded that these two residues comprise 80% of lysozyme fluorescence. Earlier workers examined the fluorescence quantum yields of lysozyme and iodine-oxidized lysozyme and concluded that Trp-108 accounts for 56% of native lysozyme fluorescence (Teichberg & Sharon, 1970). Following the suggestion of Hartdegen & Rupley (1964, 1973) and Imoto et al. (1973) that the iodine treatment only affects Trp-108, the assignment seemed unambiguous. Imoto et al. (1972) and Formoso & Forster (1975), after surveying X-ray data, concur that the Trp-62- and Trp-108-modified lysozymes suffer only subtle conformational changes. By contrast, from opposing arguments based upon multiexponential fluorescence decays (Yashinsky, 1972) and a separate kinetic analysis of lysozyme fluorescence (Elkana, 1968), it appeared that the statement that Trp-108 is the predominant fluorescence emitter in native lysozyme was not unequivocal.

Still focusing on tryptophan, Zuclich et al. (1973) explored the *phosphorescent* triplet state of tryptophan as an intrinsic spin probe in native lysozyme and demonstrated that it too was sensitive to the side-chain environment. The optically detected magnetic resonance (ODMR)[1] lines were used to examine lysozyme for evidence of multiple sites (von Schütz et al., 1974). The latter workers noted discontinuities in the zero-field parameters when plotted vs. phosphorescence wavelength, and they concluded that the phosphorescence emission of native lysozyme originates from two distinct tryptophan sites, consistent with the fluorescence observations of Imoto et al. (1972).

We have used ODMR to examine the homogeneity of the tryptophan environment in lysozyme (Rousslang et al., 1978). We have also noted that discontinuities in zero-field parameters occur in proteins containing a single tryptophan (Kwiram et

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[1] Abbreviations used: ODMR, optical detection of magnetic resonance; ADH, horse liver alcohol dehydrogenase; zfs, zero-field splittings.

Table I: Comparison of ODMR Line Positions and Line Widths for Different Tryptophan Environments[a]

substance	phosphorescence (nm)		zero-field splittings (GHz)			ODMR line widths (MHz)	
	λ_{0-0}	λ_{max}	|D − E|	|D|	|2E|	γ|D−E|	γ|2E|
ADH (Trp-15)	405.6	435.0	1.773	3.045	2.544	136	134
Gly-Trp-Gly	407.9	434.1	1.744	2.978	2.468	154	258
oxindole-108 lysozyme	409.4	437.2	1.705	3.066	2.723	110	121
azurin	410.0	438.1	1.624	3.020	2.792	42	73
ADH (Trp-314)	412.1	440.2	1.660	2.932	2.544	77	134
lysozyme (native)	413.8	442.5	1.571	2.933	2.724	83	116
oxindole-62 lysozyme	413.8	443.0	1.580	2.940	2.721	86	115

[a] All spectra at 1.4 K; solvent is 50% (v/v) ethylene glycol-water, pH 7.4 (0.05 M K_xPO_4). Excitation in all cases was at 297 nm with a 3-nm band-pass, and emission and ODMR spectra were monitored with a 1.5- or 3.0-nm band-pass. Line widths are reported for comparable sweep rates. All line positions are determined by averaging the results of increasing and decreasing frequency scans.

al., 1978). In this communication, we report the results of our study of the triplet state of tryptophan in native lysozyme and in lysozyme derivatives in which Trp-62 and Trp-108 are rendered nonphosphorescent. We feel these studies demonstrate the usefulness of phosphorescence and ODMR for examining the microenvironment of tryptophan in lysozyme. Further, our results educe Trp-108 as the primary phosphorescence species.

Experimental Section

Materials. Lysozyme was obtained from both Worthington Biochemical (2× recrystallized salt-free preparation) and Sigma (grade 1). Oxindole-62 lysozyme was a generous gift from Dr. John A. Rupley, University of Arizona, and oxindole-108 lysozyme was prepared according to Imoto et al. (1973). Azurin B (*Pseudomonas aeruginosa*) was a gift from Dr. Jon Herriott, University of Washington. Gly-Trp-Gly was purchased from Vega Fox. Horse liver alcohol dehydrogenase (ADH) from Sigma was prepared according to the procedure of Subramanian & Ross (1978). All other chemicals were reagent grade.

Sample Preparation. Protein samples, except where noted, were dissolved to a final concentration of about 10^{-5} M in ethylene glycol–H_2O (1:1 by volume) buffered to pH 7.4 in 0.1 M K_xPO_4.

Luminescence and ODMR Measurements. The phosphorescence and microwave experiments have been described in detail in earlier papers (Ross et al., 1977; Rousslang et al., 1978). For the basis of comparative phosphorescence and ODMR spectra, optical and microwave conditions were fixed, and each sample was examined in turn under nearly identical conditions (see Table I).

Results

Phosphorescence. The phosphorescence spectra of native, oxindole-62, and oxindole-108 lysozyme taken at 1.4 K are shown in Figure 1. The emission band-pass was fixed at 3 nm, and the scaling was accomplished by a dc amplifier with all other instrument settings fixed. For the excitation wavelength chosen (λ_{ex} = 297 nm), the contribution from tyrosine was absent.

Three features of the phosphorescence spectra are apparent. First, the phosphorescence of native lysozyme and oxindole-62 lysozyme coincides in wavelength and very nearly coincides in intensity as well, while oxindole-108 lysozyme shows a rather dramatic blue shift of nearly 5 nm. Second, in both oxindole-62 and native lysozyme the 0–0 vibronic band of the phosphorescence is nearly equal in intensity to the phosphorescence maximum (uncorrected spectra), whereas in oxindole-108 lysozyme the 0–0 band is relatively lower. Finally the separation between the 0–0 band and the rest of the vibronic envelope in oxindole-62 is much more distinctive than

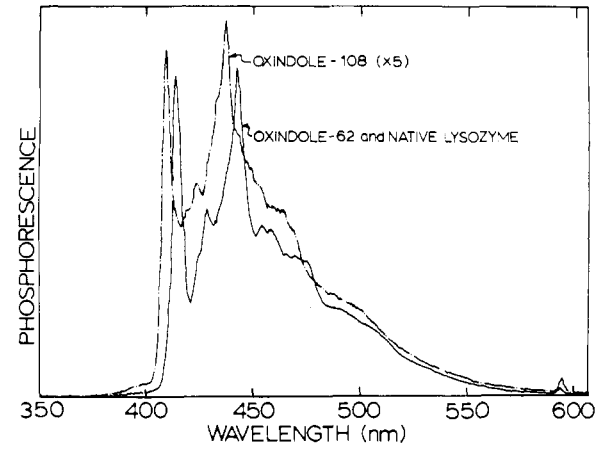

FIGURE 1: Phosphorescence spectra of oxindole-108 lysozyme (····), oxindole-62 lysozyme (—), and native lysozyme (···). Excitation = 297 nm. Phosphorescence of oxindole-108 multiplied by five (×5).

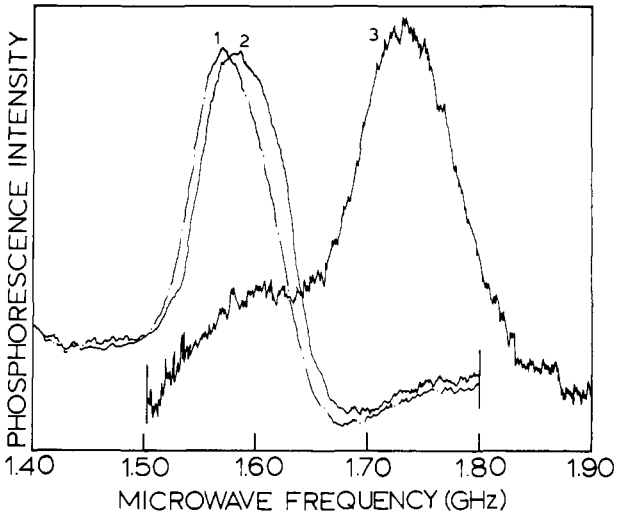

FIGURE 2: Optical detection of magnetic resonance of the |D − E| transition in native lysozyme (1), oxindole-62 lysozyme (2), and oxindole-108 lysozyme (3). Excitation wavelength = 297 nm; detection wavelength = 432 nm. Signal for oxindole-108 lysozyme multiplied by five (×5).

the same separation in oxindole-108 lysozyme. The noticeable loss of vibronic resolution, especially on the red-edge shoulders in oxindole-108, is probably attributable to a decrease in intensity and to a subsequent decrease in the signal-to-noise ratio when Trp-108 is oxidized and nonphosphorescent.

Optical Detection of Magnetic Resonance. The relative positions of the |D − E| ODMR transition in native, oxindole-62, and oxindole-108 lysozyme are shown in Figure 2. It is quite apparent that this transition in native lysozyme is only very slightly shifted when Trp-62 is oxidized, but the

transition frequency is blue-shifted by over 130 MHz when Trp-108 is oxidized to a nonphophorescent derivative. The signal-to-noise ratio in oxindole-108 lysozyme also decreases by nearly 1 order of magnitude from that observed in native or oxindole-62 lysozyme. Our results for the three lysozyme species show that, whereas the |2E| triplet splitting remains constant, the |D + E| overall splitting changes significantly, indicating that the zero-field parameter affected most is D.

A comparison of the |D − E| line width in these three forms of lysozyme reveals oxindole-62 and native lysozyme again paired and different from oxindole-108 lysozyme by about 25 MHz. In connection with our previous observations correlating an increase in the line width with a greater diversity of strongly perturbing (solvent) interactions for a luminescent amino acid in a protein (Rousslang et al., 1978), we chose additional peptides or proteins to serve as models for "exposed" or "buried" tryptophan residues. These are included in Table I as a means for comparison of tryptophan triplet parameters in diverse environments. The trends in the positions of the 0–0 band of the phosphorescence and the frequency of the |D − E| transition will be discussed below in terms of both the solvent accessibility of tryptophan in various environments and the polarizability of the medium that determines the electric and magnetic environment of the tryptophan triplet.

Discussion

Model. It is still difficult, even with the combined extensive efforts of numerous research groups, to assign unequivocally those tryptophans in lysozyme responsible for emission. Consequently, our new observations on the phosphorescence and ODMR characteristics of tryptophan in lysozyme and other peptides and proteins will depend heavily on the composite model of lysozyme luminescence that has emerged from previous work.

As we stated earlier, there is strong evidence that the principal (fluorescent) radiative sites in lysozyme are Trp-62 and Trp-108, with somewhat less than 20% of the fluorescence arising from the other residues, Trp-28, Trp-63, Trp-111, and Trp-123. Even though each residue does not necessarily phosphoresce equally—just as they do not fluoresce equally—and furthermore, although *those species responsible for fluorescence do not necessarily comprise those responsible for the phosphorescence* (and subsequently, the ODMR spectra), we will use and then test the following assumptions. (1) The crystal structure data demonstrate adequately that Trp-62 is at the cleft of the active site of lysozyme and therefore is a surface residue and Trp-108, although also at the active site, is effectively "buried" in the sense that the aromatic side chain is sandwiched by adjacent groups and in an environment where solvent access is severely restricted. (2) The principal phosphorescent species in lysozyme are Trp-62 and Trp-108. (3) Relative to Gly-Trp-Gly[2] in aqueous solvent, buried tryptophans have red-shifted phosphorescence and exposed residues mimic Gly-Trp-Gly. (4) Relative to Gly-Trp-Gly in aqueous solvent, (a) Buried tryptophans have red-shifted ODMR lines (|D − E| only) and exposed residues have ODMR lines approaching free tryptophan[3] and (b) ODMR line widths narrow as the tryptophan residues become less solvent accessible.

Phosphorescence. From inspection of Figure 1, it is apparent that oxidation of Trp-62 does not shift the spectrum nor reduce the intensity severely from that of native lysozyme at the same concentration. Since both spectra are considerably red-shifted from Gly-Trp-Gly (Table I), emission is apparently dominated by relatively buried residues in both cases. Further evidence for this conclusion is given by the phosphorescence spectrum of oxindole-108 lysozyme, in which emission maxima approach those of Gly-Trp-Gly. Apparently, when Trp-108 is oxidized, the remaining emission is reduced fivefold in intensity and is presumably due to the surface residue Trp-62. Because of the identical maxima in oxindole-62 and native lysozyme and the above arguments, it seems that Trp-108 dominates the phosphorescence in native lysozyme, if we assume (a) that the oxidation of Trp-62 does not severely alter the structure of lysozyme and hence the microenvironment of Trp-108 (Imoto et al., 1972; Formoso & Forster, 1975) and (b) that, again, the contributions from other residues are not dominant when Trp-62 is oxidized. Fluorescence studies demonstrate that in native lysozyme, Trp-63 transfers energy to Trp-62 efficiently (Imoto et al., 1972); it is, however, entirely possible that when Trp-62 is rendered nonluminescent, Trp-63 emerges as a luminescent species. There is also evidence for singlet–singlet energy transfer from Trp-108 to Trp-62 (Formoso & Forster, 1975). The fact that the 20% blue-shifted phosphorescence intensity remaining in oxindole-108 lysozyme is not observed in the spectra of native lysozyme may be a consequence of triplet–triplet energy transfer from Trp-62 to Trp-108. That Trp-108 can be an acceptor on the triplet level, whereas it is a donor on the singlet level, is simply a consequence of the observation that buried tryptophan residues with a red-shifted phosphorescence have a blue-shifted fluorescence. Similarly, in oxindole-62 lysozyme, Trp-63 may emerge as the triplet–triplet donor since the oxidation eliminates the deactivating interaction with Trp-62. These specific issues remain to be explored.

Zero-Field Splittings (zfs). In our recent investigations on tryptophan-containing proteins, we have noted that in aqueous solvent, the relatively solvent-accessible tryptophan residues have a larger |D − E| splitting than partially or wholly buried tryptophan residues (Ross et al., 1977; Rousslang et al., 1978). In Table I, it is apparent that native lysozyme and lysozyme without Trp-62 have similar |D − E| line positions and fall in the same vicinity (relative to other table entries) as |D − E| of tryptophan in azurin (*Pseudomonas aeruginosa*) (Ugurbil et al., 1977; Rousslang et al., 1978) or Trp-314 in liver alcohol dehydrogenase (ADH).[3] Since Trp-314 in ADH is known from crystal structure to be a buried residue (Eklund et al., 1974; Bränden et al., 1975) and the single tryptophan in azurin is also known from X-ray data to be completely buried (Adman et al., 1978), the ODMR signal of oxindole-62 lysozyme apparently arises from a tryptophan residue with a similar solvent accessibility or similar polarity of environment, i.e., Trp-108.

Similarly, the |D − E| splitting of tryptophan in oxindole-108 lysozyme falls in the same region as Trp-15 in ADH, reported to be an exposed residue (Eklund et al., 1974), or tryptophan in Gly-Trp-Gly (Deranleau et al., 1978), presumably a model of a solvent-accessible environment for tryptophan. The signal in oxindole-108 lysozyme probably arises from one of the exposed tryptophan residues on the protein surface at the active site—Trp-62 or Trp-63—and, consistent with our hypothesis, the signal is probably attributable to Trp-62. Also, the sig-

[2] An appropriate model for "free" or solvent-accessible tryptophan that is still incorporated into a polypeptide chain is very difficult to choose. Consistent with our previous work on polypeptide chains (Deranleau et al., 1978), we have again chosen Gly-Trp-Gly as a reasonable model of a peptide with solvent-available tryptophan.

[3] If we consider the parameter D, then Trp in azurin would seem to belong in the "exposed" rather than in the "buried" group. Further examples will have to be investigated before a rigorous categorization is possible.

nal-to-noise ratio of this ODMR signal in oxindole-108 is lower than the same transition in oxindole-62 by nearly the same factor as the ratio of their phosphorescence intensities, indicating that if we are monitoring primarily the signals from Trp-62 and Trp-108 in native lysozyme, the assignments we shall make from the ODMR zfs match precisely the predictions we would make based upon the phosphorescence results.[4]

Of course, having the doubly oxidized lysozyme species in which both Trp-62 and Trp-108 are rendered nonphosphorescent (Formoso & Forster, 1975) would obviate the need in our discussions to exclude the nonzero contributions to the ODMR lines arising from any or all of the other buried tryptophans. We have been unable to obtain the doubly oxidized derivative, so our desire to include the phosphorescence and ODMR results on this form of lysozyme has been frustrated.

ODMR Line Widths. In a previous paper (Rousslang et al., 1978) we outlined our experimental results on the overall tryptophan ODMR line width in proteins and showed that it could be related to the degree of solvent availability of the phosphorescent residues. We concluded that when solvent was present and able to approach the luminescent residue, it dominated the line width. We also supposed that an exposed residue would experience a wider range of strongly perturbing solvent interactions and therefore have a broader ODMR line than a buried residue. (The effect of charged residues should also be considered.)

The line width increases in going from native or oxindole-62 lysozyme to oxindole-108 lysozyme. This is true for either the $|D - E|$ line width or the $|2E|$ line width. Again, if our assignment of the ODMR lines to the tryptophan residues is correct, then Trp-108 (i.e., in oxindole-62 or native lysozyme) not only dominates the phosphorescence and ODMR spectra but also is responsible for the resonance line at 1.58 GHz in oxindole-62 lysozyme (or 1.57 GHz in native lysozyme) *consistent with the fact that the line is narrow and like that of a buried residue.* The broader line observed in oxindole-108 lysozyme is probably associated with Trp-62 or Trp-63, which experience a broader distribution of solvent interactions than Trp-108.

We suspect that the narrowest line we have observed for tryptophan in a protein—that for the single tryptophan in azurin—represents a lower limit to the line width for tryptophan in a native protein. The much broader lines in lysozyme may arise from more than just a consideration of the solvent accessibility of the tryptophan sites responsible for the phosphorescence. Although Trp-108 is minimally exposed to solvent (J. C. Hodsdon and L. H. Jensen, personal communication), it is possible that the degree of solvent exposure alone will not explain the comparatively broader $|D - E|$ line observed in oxindole-62 lysozyme vs. azurin (86 vs. 42 MHz). Besides the intermolecular interactions with the solvent, there are also the intramolecular interactions within the protein. Each protein conformer will give rise to a different environment for a given tryptophan residue. In other words, there may be less diversity in the conformers of azurin than in the conformational forms of lysozyme. Alternately, the tryptophan emission and hence contribution to the ODMR line from the four other tryptophans in lysozyme are probably not zero, and the fact that azurin contains only one tryptophan residue may entirely account for the narrower lines in this protein.

On this note, a rather interesting comparison is made by examining the $|D - E|$ line width of the *two* phosphorescence-resolved (Purkey & Galley, 1970) tryptophan residues in ADH. In agreement with Zuclich et al. (1973), Trp-15, an exposed residue, has nearly double the line width of Trp-314, a buried residue, yet Trp-314 still has almost double the line width of tryptophan in azurin. There are at least two possible explanations. It may be that the wider lines of Trp-314 are due to similar but not identical contributions from each Trp in the two subunits of the ADH dimer, especially since the two tryptophans are close enough to participate (asymmetrically) in a hydrogen bond (Bränden et al., 1975). On the other hand, the local interactions experienced by a tryptophan residue in the interior of a protein may be influenced strongly by the rather large dipolar interaction of the aromatic ring with one or more adjacent peptide bonds or, in the case of ADH, a dipolar interaction between the two hydrogen-bonded Trp-314 residues. Thus, the line width is not simply a function of solvent accessibility but in some cases may also be strongly influenced by local dipolar and hydrogen-bonding interactions due to the peptide chain itself. Restricted solvent accessibility may be a neccessary condition for narrow lines, but it is not a sufficient condition.

Naturally, it will always be far less ambiguous to interpret line widths and line positions in proteins containing a single tryptophan. Nevertheless, we feel that both the phosphorescence and ODMR data presented here provide unmistakable evidence that the selective experiments performed on a multitryptophan protein can give insight into specific sites. In particular, the evidence that only two residues in lysozyme contribute the majority of the fluorescence *and* phosphorescence intensity may lead to valuable information on the mode of tryptophan fluorescence quenching in proteins, since the other four residues are apparently deactivated nonradiatively. The mechanism by which the latter occurs remains to be investigated.

Although fluorescence has been used much more extensively than phosphorescence to investigate biomolecular phenomena, there is valuable information available from examination of the triplet state. Perhaps the preponderance of triplet-state studies done in rigid media (mixtures of ethylene glycol or glycerol and water) has hidden the fact that phosphorescence in some proteins can be resolved above glass transition temperatures and in some cases even at room temperature (Saviotti & Galley, 1974). Unfortunately, loss of resolution usually accompanies high-temperature phosphorescence studies. On the other hand, there is reasonable evidence that proteins do retain the dominant elements of native structure at the temperatures required for rigid glass formation of the solvent (Douzou, 1977).

Though ODMR experiments involving tryptophan currently necessitate pumped helium temperatures, it should be possible to eventually examine the triplet-state populations at temperatures approaching room temperature. The experiment will have to overcome both the triplet collisional (i.e., diffusion controlled) deactivation rate and the spin–lattice relaxation rates within the triplet state, a prospect which seems entirely feasible.

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[4] One might argue that a residual peak due to Trp-62 should appear at 1.75 GHz in the native lysozyme spectrum. However, if energy transfer between Trp-62 and Trp-108 occurs in native lysozyme (see Phosphorescence), such a peak would not be expected. On the other hand, the presence of a weak signal at 1.75 GHz cannot be excluded at this time.

of oxindole-62 lysozyme.

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Lipid Lateral Diffusion by Pulsed Nuclear Magnetic Resonance†

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ABSTRACT: The temperature and hydration dependences of lipid lateral diffusion in model membrane–D_2O multilayers of dipalmitoyl (DPL), dimyristoyl (DML), dilauryl (DLL), and egg yolk (EYPC) lecithins were measured by using pulsed gradient proton NMR spin–echo techniques. Oriented samples were used to minimize anisotropic dipolar interactions and permit formation of a spin–echo. A general discussion of the technique and of the possible errors is included. Significantly lipid lateral diffusion is hydration dependent over the range studied (15–40% D_2O, w/w), varying in DPL over this range, for example, by a factor of 2. For the saturated lipids at the same hydration and temperature, diffusion decreases monotonically as the chain length increases. At a constant hydration of 20% D_2O (w/w), the activation energies (kcal/mol) are as follows: EYPC, 9.0 ± 0.4; DLL, 9.4 ± 0.2; DPL, 18.6 ± 1.3; DML, 15.2 ± 0.3. The results tend to be larger, by factors of 2–5, than the earlier ESR spin-label results, the differences being attributable in part to the differences in hydration and to the absence of probe effects in this work. The value of 5×10^{-8} cm^2/s for DPL (40% D_2O) at 42 °C is slightly larger than the usually reported spin-label value of 2×10^{-8} and than the fluorescence photobleaching recovery measurements which give typically 1×10^{-8} cm^2/s. The results agree with recent photo-spin-label measurements. Cholesterol in small amounts (less than 10 mol %) in DPL *increases* lipid diffusion; its presence in larger concentrations decreases diffusion.

Many fundamental processes in membranes and cells require molecular motions of the membrane components (Singer & Nicolson, 1972; Edidin, 1974; Frye & Edidin, 1970; Wu et al., 1977; Shimshick & McConnell, 1973; Linden et al., 1973; Crick, 1970). In particular, lateral diffusion of lipids and of proteins is important in embryological development (Crick, 1970), in cell fusion (Frye & Edidin, 1970; Wu et al., 1977), in membrane phase separations (Shimshick & McConnell, 1973), and in membrane transport processes (Linden et al., 1973). X-ray diffraction (Levine, 1972), differential calorimetry (Chapman, 1975; Melchior & Steim,

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