# Structural Effects of Myelin Proteolipid Apoprotein on Phospholipids: A Raman Spectroscopic Study†

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ABSTRACT: Raman spectroscopy indicates that the myelin proteolipid apoprotein (PLA) perturbs the configuration and thermal behavior of the acyl chains of phospholipids. Spectral data in the 1000–1150-$cm^{-1}$ (C–C stretching) region for a dimyristoyllecithin (DML)/PLA recombinant indicate that, below the lipid order–disorder transition, the number of DML acyl chain trans conformers is reduced in the presence of PLA. Above the transition, the DML acyl chains are prevented by PLA from completely attaining the extensive gauche conformation. Spectra in the 2800–3000-$cm^{-1}$ (C–H stretching) region for the DML/PLA recombinant indicate that, at temperatures above the cooperative calorimetrically observed order–disorder transition (Curatolo, W., Sakura, J. D., Small, D. M., & Shipley, G. G. (1977) *Biochemistry 16,* 2313), the DML acyl chains, on the average, possess some solid character. This solid character decreases as the temperature is increased in the liquid crystalline state, but is still observable 18 °C above the DML phase transition temperature. The temperature dependence of Raman spectra of egg yolk lecithin (EYL)/PLA recombinants in the 2800–3000-$cm^{-1}$ region is more complex than that of the DML/PLA recombinants. In the presence of PLA, the EYL order–disorder transition becomes extremely broad (−26 to +15 °C). Superimposed on the EYL transition is a smaller transition at +12 °C which is shown by differential scanning calorimetry to involve a cooperative structural change.

Proteolipids, operationally defined as protein–lipid complexes which are soluble in chloroform–methanol mixtures, have been observed in a variety of neural (Folch & Lees, 1951) and nonneural tissues (Folch-Pi & Sakura, 1976), and in subcellular preparations such as mitochondria (Cattel et al., 1971; Tzagoloff & Meagher, 1972) and sarcoplasmic reticulum (MacLennan et al., 1972). The myelin proteolipid apoprotein (PLA),[1] which is the delipidated form of the white matter proteolipid first isolated by Folch & Lees (1951), is the major protein of myelin (Autilio et al., 1964; Gonzalez-Sastre, 1970). This unusually hydrophobic protein, which is soluble in organic solvents, possesses chemical and physical properties which suggest that it occupies a position deeply embedded in the hydrocarbon interior of myelin multilayers. PLA has a primary structure consisting of greater than 50% apolar amino acids and possesses covalently bound fatty acids (Gagnon et al., 1971; Folch-Pi & Stoffyn, 1972). Freeze-fracture electron micrographs of myelin (Pinto da Silva & Miller, 1975) and of recombinants of the N-2 protein[2] of human myelin with dipalmitoyllecithin (Papahadjopoulos et al., 1975) exhibit particles in the fracture plane, characteristic of protein which is deeply embedded in the hydrocarbon region of the bilayer. Membrane-reconstitution studies (Racker, 1975, 1976) suggest that proteolipids in some membranes, e.g., sarcoplasmic reticulum, may act as ionophores.

A recent calorimetric study of recombinants of PLA with dimyristoyllecithin (DML) has shown that, in the presence of PLA, a population of DML molecules undergoes a perturbed order–disorder transition (Curatolo et al., 1977). Thermodynamic arguments indicated that this population, referred to as "boundary" lipid (after Jost et al., 1973), must possess partially disordered acyl chains below the transition or partially ordered ("bound") acyl chains above the transition, or both. Thermodynamic measurements are insufficient to distinguish between these possibilities.

Raman spectroscopy has provided a sensitive measure of hydrocarbon chain conformation in monoglycerides and cardiolipin (Larsson & Rand, 1973), in saturated and unsaturated lecithins (Lippert & Peticolas, 1971; Bulkin & Krishnamachari, 1972; Lis et al., 1975; Spiker & Levin, 1975; Verma & Wallach, 1975; Mendelsohn et al., 1975), in phospholipid/protein recombinants (Lis et al., 1976a,b; Verma & Wallach, 1976a), in plasma membranes (Wallach & Verma, 1975; Verma et al., 1975; Schmidt-Ullrich et al., 1976; Verma & Wallach, 1976b,c), and in sarcoplasmic reticulum (Milanovitch et al., 1976). In this paper we report Raman scattering studies on recombinants of the proteolipid apoprotein and dimyristoyl- or egg yolk lecithin (EYL). Our results provide information about the absolute configurational states of protein-associated lipids above and below their order–disorder transition temperatures.

## Materials and Methods

*Myelin Proteolipid Apoprotein (PLA).* Proteolipids were extracted from freshly dissected bovine white matter and delipidated by extensive dialysis against chloroform/methanol mixtures (Folch-Pi & Stoffyn, 1972). Chemical analysis of PLA showed it to contain 0.8% phosphorus and 3.1% fatty acid.

*Egg Yolk Lecithin (EYL).* EYL was isolated and purified according to Litman (1973).

*Dimyristoyllecithin (DML).* Glycerylphosphorylcholine

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[1] Abbreviations used: PLA, proteolipid apoprotein; DML, dimyristoyllecithin; DSC, differential scanning calorimetry; EYL, egg yolk lecithin; ESR, electron spin resonance spectroscopy; DPL, dipalmitoyllecithin.

[2] The N-2 protein of human myelin (Gagnon et al., 1971) possesses chemical and physical properties similar to those of PLA of bovine myelin. This protein is also known as lipophilin (Boggs et al., 1976).

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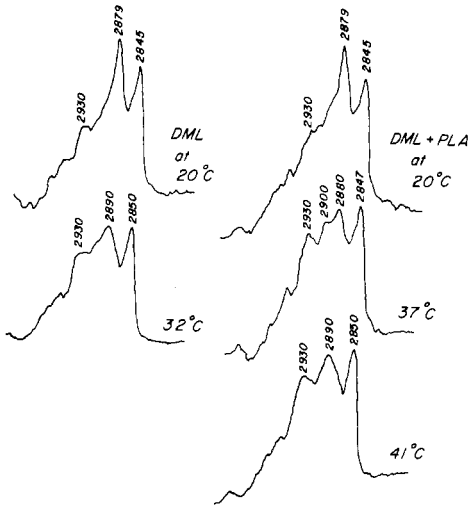


FIGURE 1: Raman spectra of DML (left) and a 20:1 (w/w) DML/PLA recombinant (right) in the 2800-3000-$cm^{-1}$ (C-H stretching) region.

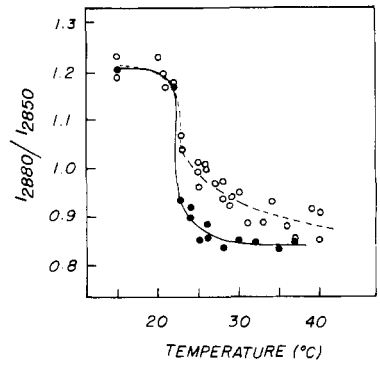


FIGURE 2: $[I_{\sim 2880}/I_{\sim 2850}]$ as a function of temperature for DML (solid line) and a 20:1 (w/w) DML/PLA recombinant (dashed line).

was prepared from EYL according to Chadha (1970) and was reacted with myristic anhydride to form DML by the method of Cubero Robles & Van Den Berg (1969). DML was purified by chromatography on silicic acid and shown to be greater than 99% pure by thin layer chromatography. Myristic acid was purchased from Nu-Chek Prep (Elysian, Minn.).

*DML/PLA Recombinants.* Stock solutions of PLA and DML (or EYL) in chloroform-methanol (2:1, v/v) were mixed in appropriate proportions and dried under a stream of $N_2$ in acid-washed constricted glass tubes. After overnight desiccation under vacuum, the samples were hydrated with $H_2O/D_2O$ (50:50), and the tubes sealed. Equilibration was achieved by centrifuging the samples repeatedly through the constriction at 45 °C for 3 h. The DML (or EYL) concentration in all samples was either 15 mg/mL or 200 mg/mL. Samples for differential scanning calorimetry were prepared similarly to those for Raman spectroscopy, by mixing phospholipid and PLA in chloroform/methanol (2:1, v/v) solution. The solutions were added to Perkin-Elmer DSC pans (50-$\mu$L capacity), dried down under $N_2$, and desiccated under vacuum overnight. Doubly distilled water was added, and the pans were sealed and held at 45 °C for 3 h.

*Raman Spectra.* Raman spectra were recorded using a Ramalog 4 Raman spectrometer (Spex Industries, Metuchen, N.J.) interfaced to an Interdata (Model 70) computer. An $Ar^+$ laser (Spectra Physics Model 164), tuned to 488 nm, was used as an excitation source at a power of 300 mW. Detection and processing of the scattered radiation were as previously described (Verma et al., 1975). Samples were held in sealed Kimex capillaries (0.9-1-mm internal diameter) in a Harney-Miller cell. Temperature was controlled by a flow of warm or cold nitrogen and measured with a telethermometer. Samples were equilibrated at the desired temperature for 20 min with the laser beam occluded, followed by 5 min in the beam before recording spectra.

*Differential Scanning Calorimetry (DSC).* Calorimetric data were obtained on a Perkin-Elmer DSC-2 differential scanning calorimeter, at a scanning rate of 5 °C/min, at a sensitivity of 1.0 mcal/s.

## Results

*I. DML/PLA Recombinants.* C-H Stretching Region. The Raman spectrum of DML in the 2800-3000-$cm^{-1}$ region (Figure 1) exhibits a number of bands arising from both fundamental C-H stretching vibrations and their interactions with H-C-H deformation overtones of the phospholipid acyl chains (Verma & Wallach, 1977a; Synder et al., 1978). The intense bands at 2845-2850 $cm^{-1}$ and 2879-2890 $cm^{-1}$ have been assigned to methylene symmetric and asymmetric C-H stretching, respectively (Larsson, 1973; Larsson & Rand, 1973; Spiker & Levin, 1975; Verma & Wallach, 1977a). Another band around 2930 $cm^{-1}$ probably includes contributions from symmetric C-H stretching from methylenes and terminal methyl groups of the acyl chains (Bunow & Levin, 1977; Verma & Wallach, 1977a). The ratio of the intensity of the 2880-2890-$cm^{-1}$ band to that of the 2845-2850-$cm^{-1}$ band has been shown to be sensitive to the order-disorder transition of dipalmitoyllecithin (Brown et al., 1973), as well as DML and EYL (Verma & Wallach, 1976a). In addition, shifts in the observed frequencies of these bands occur upon heating through the order-disorder transition: from 2845 $cm^{-1}$ and 2880 $cm^{-1}$ to 2850 $cm^{-1}$ and 2890 $cm^{-1}$, respectively. Representative spectra of DML taken below (20 °C) and above (32 °C) the order-disorder transition temperature illustrate the typical temperature-dependent changes (Figure 1).

Spectra of the DML/PLA recombinant (20:1, w/w) at temperatures below (20 °C) and above (37 °C) the DML order-disorder transition appear qualitatively the same as spectra of DML alone, except that the 2880-$cm^{-1}$ band does not shift to 2890 $cm^{-1}$ in the temperature range 24-37 °C, as is normally observed with DML. However, by 41 °C, the peak has shifted to 2890 $cm^{-1}$ for the recombinant.

The ratio of the intensity of the 2880-2890-$cm^{-1}$ band to that of the 2845-2850-$cm^{-1}$ band $[I_{\sim 2880}/I_{\sim 2850}]$ is plotted as a function of temperature for a 20:1 (w/w) DML/PLA recombinant and for DML alone in Figure 2. For DML alone the intensity ratio shows a sharp decrease at ~22 °C, which is 1-2 °C lower than the transition temperature of DML observed by calorimetric methods (Ladbrooke & Chapman, 1969; Hinz & Sturtevant, 1972). This apparent depression in transition temperature is due to localized heating of the sample by the laser beam (Verma & Wallach, 1976a). The DML/PLA recombinant (20:1, w/w) shows clearly an initial sharp decrease in intensity ratio at 22 °C followed by a slower decline.

C-C Stretching Region. Phospholipids exhibit bands in the 1000-1150-$cm^{-1}$ region assigned to C-C stretching vibrations which have been shown to be sensitive to hydrocarbon chain conformation (Lippert & Peticolas, 1971; Spiker & Levin, 1975). The major features in this region are two intense bands at 1064 and 1130 $cm^{-1}$, assigned to vibrations of the trans conformation of the acyl chains, and a broader band at 1095 $cm^{-1}$ assigned to vibrations of acyl chain segments containing randomly oriented C-C bonds (Figure 3). For DML at tem-

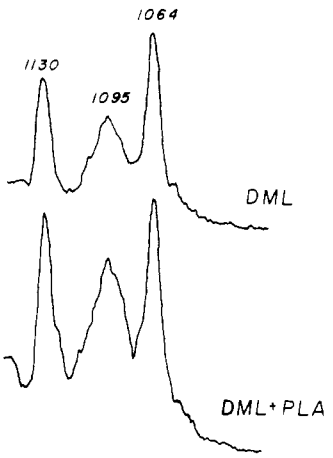


FIGURE 3: Characteristic Raman spectra of DML and a 20:1 (w/w) DML/PLA recombinant in the 1000–1150-cm$^{-1}$ (C–C stretching) region, at 20 °C.

TABLE I: Intensity Data for Raman C–C Stretching Vibrations of Phospholipid and Phospholipid/PLA Recombinants.

| Sample | $T$ (°C) | $[I_{1064}/I_{1095}]$ | $S^a$ |
|---|---|---|---|
| DML | 20 | 2.45 | 0.98 (±2%) |
| | 22 | 1.46 | 0.53 (±4%) |
| | 30 | 0.30 | 0 (±2%) |
| | 35 | 0.28 | 0 (±2%) |
| DML/PLA (20:1, w/w) | 20 | 1.80 | 0.68 (±7%) |
| | 22 | 1.80 | 0.68 (±7%) |
| | 30 | 1.20 | 0.41 (±3%) |
| | 35 | 0.48 | 0.08 (±25%) |

[a] $S$ is a conformational parameter derived to normalize intensity data. See Results section for calculation of this parameter.

peratures below that of the order–disorder transition, the ratio $[I_{\sim1064}/I_{\sim1095}]$ is greater than 1.0. At the order–disorder transition temperature this ratio falls sharply to below 1.0. $[I_{\sim1064}/I_{\sim1095}]$ ratios are presented in Table I for both DML and a DML/PLA recombinant (20:1, w/w) at selected temperatures. To simplify comparison of intensity ratios between different samples, we have followed Gaber & Peticolas (1977) in deriving a conformational parameter $S$:[3]

$$S = \frac{(I_{1064}/I_{1095})_{\text{sample}} - (I_{1064}/I_{1095})_{\text{liquid crystalline PC}}}{(I_{1064}/I_{1095})_{\text{gelPC}} - (I_{1064}/I_{1095})_{\text{liquid crystalline PC}}}$$

This conformational parameter varies between 0 and 1.0, where zero defines the acyl chain configuration of liquid-crystalline (melted-chain) lecithin, but does not imply a truly random state. The maximum value, 1.0, defines the lecithin acyl chains in the gel (rigid-chain) state. Below the DML order–disorder transition temperature, the DML/PLA recombinant exhibits a lower value of $S$ than DML alone (Table I), indicating perturbation of DML acyl chain conformation by the protein. Above the phase transition temperature, the opposite is true: the DML/PLA recombinant exhibits a higher value of $S$ than DML alone, suggesting that some or all of the DML acyl chains in the recombinant still possess significant trans conformation.

*II. EYL/PLA Recombinants.* C–H Stretching Region. The

[3] This conformational parameter is derived only to normalize our data and is not directly related to the order parameter $S$ derived from electron spin resonance and deuterium magnetic resonance spectroscopy. Liquid crystalline PC and gel PC refer to lecithin above and below the order–disorder transition, respectively.

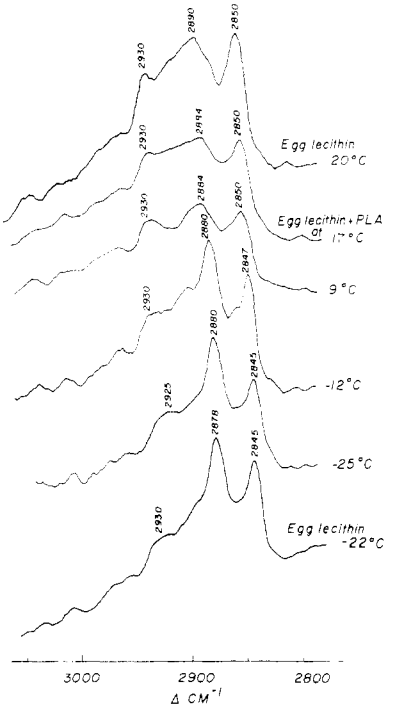


FIGURE 4: Raman spectra in the 2800–3000-cm$^{-1}$ (C–H stretching) region of EYL at 20 and −22 °C, and a 20:1 (w/w) EYL/PLA recombinant at 17, 9, −12, and −25 °C.

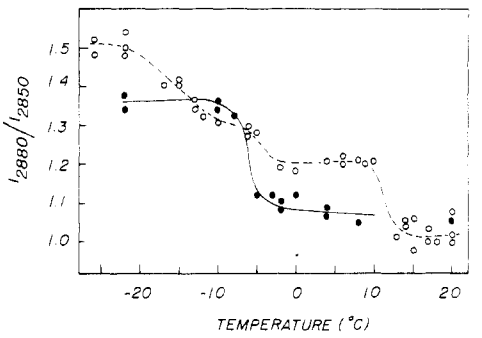


FIGURE 5: $[I_{\sim2880}/I_{\sim2850}]$ as a function of temperature for EYL (solid line) and a 20:1 (w/w) EYL/PLA recombinant (dashed line).

Raman spectra of EYL and an EYL/PLA recombinant (20:1, w/w) in the 2800–3000-cm$^{-1}$ region (Figure 4) exhibit the same general features as the spectrum of DML in this region. The variation with temperature of $[I_{\sim2880}/I_{\sim2850}]$ for an EYL-PLA recombinant (20:1, w/w) differs from that of EYL alone (Figure 5). In the presence of PLA, the EYL transition centered at approximately −7 °C becomes very broad, spanning the range −20 °C to +15 °C. $[I_{\sim2880}/I_{\sim2850}]$ for the recombinant is significantly greater than that for EYL alone at temperatures below that of the EYL transition, while at high temperatures (>15 °C) both EYL and the EYL/PLA recombinant reach nearly the same minimum ratio. In addition, a sharp discontinuity in $[I_{\sim2880}/I_{\sim2850}]$ appears at about 12 °C in the EYL/PLA recombinant (see Figure 5).

Differential Scanning Calorimetry (DSC). To ascertain whether the 12 °C transition observed in the Raman C–H stretching region for EYL/PLA recombinants represents a cooperative structural change between long-lived conformational states, scanning calorimetry of EYL and an EYL/PLA recombinant (15:1, w/w) was performed. To avoid interference from the large ice–water transition peak at 0 °C, samples were supercooled to −8 °C (without the freezing of water) and then

immediately heated at 5 °C/min (Figure 6a,c). In the temperature range 0–30 °C the order–disorder transition of pure EYL is not observed when this protocol is followed (Figure 6c). When an EYL/PLA (15:1, w/w) recombinant is subjected to this thermal protocol (Figure 6a), the descending portion (high temperature side) of a broad peak is observed between −3 and +15 °C. Superimposed on this large broad peak is a small transition with peak maximum at approximately 12 °C. This small peak is sharper during a cooling run, and supercools, its maximum being at 5 °C (Figure 6b).

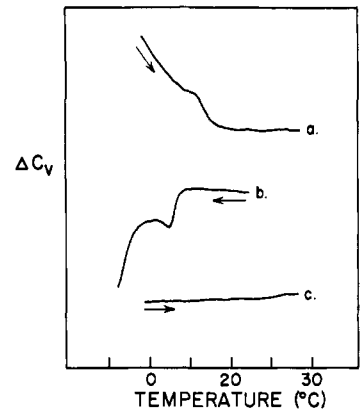


FIGURE 6: Differential scanning calorimetry traces of (a) a 15:1 (w/w) EYL/PLA recombinant (heating run); (b) 15:1 (w/w) EYL/PLA recombinant (cooling run); (c) EYL. Samples contained approximately 10 mg of EYL and were run at a scanning rate of 5 °C/min. Heating runs were performed as follows: the sample was supercooled to −8 °C and immediately heated at 5 °C/min to avoid freezing the water in the sample. This allowed the visualization of part of the broad EYL/PLA (15:1, w/w) transition peak.

## Discussion

Raman spectroscopic data indicate that the myelin proteolipid apoprotein perturbs the configuration and thermal behavior of the acyl chains of phospholipids. A previous scanning calorimetry study (Curatolo et al., 1977) showed that the addition of PLA to DML bilayers modified DML thermal behavior, causing the appearance of a broad calorimetric peak, maximal at 2 °C above the temperature of the DML order–disorder transition. For the DML/PLA recombinants, the Raman spectroscopy data obtained in the 1000–1150-$cm^{-1}$ (C–C stretching) region and the 2800–3000-$cm^{-1}$ (C–H stretching) region confirm this observation and provide information on the molecular configurations of the PLA-associated acyl chains. The conformational parameter $S$ (normalized intensity ratio)[3] indicates that at 20 °C, a temperature below that of the DML order–disorder transition, the DML acyl chains in the recombinant ($S$ = 0.68) possess more gauche conformation on the average than those of DML alone ($S$ = 0.98).[4] Thus some DML acyl chains are prevented by PLA from crystallizing into their usual trans form below the DML transition temperature. At 30 °C, a temperature above the DML order–disorder transition, the conformational parameter for the recombinant ($S$ = 0.41) is larger than that for DML alone ($S$ = 0.0), indicating that the phospholipid acyl chains in the recombinant possess more trans configuration on the average than would be found in pure liquid-crystalline DML. Thus, the recombinant contains a population of DML molecules whose acyl chains have not been completely melted to the liquid-crystalline state. Although we cannot rule out the possibility that PLA induces, through long range interactions, perturbations to all DML molecules in the recombinant, it is probable that PLA sterically constrains a population of "boundary" DML acyl chains from attaining the extensive trans conformation below the transition temperature or the extensive gauche conformation above the transition temperature.

Raman spectra of phospholipids in the 2800–3000-$cm^{-1}$ (C–H stretching) region provide information on acyl chain organization (Larsson & Rand, 1973; Verma et al., 1975; Verma & Wallach, 1977a; Gaber & Peticolas, 1977). For DML, the intensity ratio $[I_{\sim 2880}/I_{\sim 2850}]$ undergoes a sharp change at 22 °C corresponding to the acyl chain order–disorder transition (Figure 2). The DML/PLA recombinant (20:1) w/w), on the other hand, exhibits an initial sharp decrease in $[I_{\sim 2880}/I_{\sim 2850}]$ at ~22 °C, followed by a broader decline. This suggests that two populations of DML exist in the presence of PLA (at 20:1, w/w): one whose acyl chain melting is relatively unperturbed by the protein, and another whose acyl chain thermal behavior is affected by its association with PLA. Similar behavior has been observed for DML/PLA recombinants by differential scanning calorimetry (Curatolo et al., 1977). Raman spectra of the recombinant in the temperature range 24–37 °C (Figure 1) show the presence of the 2880-$cm^{-1}$ feature, while spectra of DML alone exhibit the band at 2890 $cm^{-1}$. It has been shown previously that the band at 2880 $cm^{-1}$ is due to the interaction (enhanced by Fermi resonance) of methylene C–H stretching fundamentals and H–C–H deformation overtones, which vanishes in the melted-chain state (Verma & Wallach, 1977a; Snyder et al., 1978). The 2890-$cm^{-1}$ band which remains in the C–H stretching region above the order–disorder transition is a true asymmetric mode of the acyl chain methylenes. The presence of the 2880-$cm^{-1}$ band over the temperature range 24–37 °C in the DML/PLA recombinant indicates that some of the DML acyl chains have not melted, and the resonance-enhanced 2880-$cm^{-1}$ feature arising from this population dominates the spectrum in the region 2865–2900 $cm^{-1}$. This effect is also evidenced in the higher $[I_{\sim 2880}/I_{\sim 2850}]$ ratio for the recombinant in this temperature range (Figure 2).

Differential scanning calorimetry of a DML/PLA recombinant (20:1, w/w) (Curatolo et al., 1977) showed analogous behavior, with the important difference that the calorimetric transition of "boundary" DML was complete by 32 °C. Model-building arguments were used to show that the "boundary layer" extends out from the protein in 3–4 concentric annuli *at the phase transition,* but no estimate of the size or state of the "boundary layer" at higher temperatures could be derived. The thermal behavior of the DML/PLA recombinant in the Raman C–H stretching region (Figure 2) indicates that, at temperatures above that at which the cooperative calorimetrically observed transition is complete, the DML acyl chains *still possess some solid character.* In addition, the difference in $[I_{\sim 2880}/I_{\sim 2850}]$ between the recombinant and pure DML decreases with increasing temperature, indicating that the effective size of the "boundary layer" may decrease with increasing temperature in the liquid crystalline state. Such a temperature-dependent conformation for the acyl chains in a lecithin/PLA recombinant above the order–disorder transition has also been observed using electron spin resonance spectroscopy (Boggs et al., 1976).

At temperatures below that of the DML order–disorder transition, no significant difference in $[I_{\sim 2880}/I_{\sim 2850}]$ is ob-

[4] These data cannot be used to quantitate the number of trans and gauche bonds in the "boundary" (PLA-associated) DML acyl chains since, at 20:1 (w/w) DML/PLA, both "free" and "boundary" populations of DML are present (Curatolo et al., 1977). The Raman intensity data report on the average of these populations.

served between the recombinant and DML alone (Figure 2). This indicates that the acyl chains in the recombinant have solidified, and do not possess any less lateral chain interaction than pure DML in the gel state. Intensity data in the 1000–1150-$cm^{-1}$ (C–C stretching) region (Table I) indicate, however, that the acyl chains in the "boundary layer" about PLA are frozen into configurations that are not all-trans.

The behavior of the EYL/PLA recombinant in the 2800–3000-$cm^{-1}$ (C–H stretching) region (Figure 5) is more complex than that of the DML/PLA recombinant, as might be expected from the heterogeneous acyl chain distribution present in EYL. In the presence of PLA, the EYL transition becomes extremely broad and an additional transition occurs at 12 °C. The differential scanning calorimetry data show that the latter discontinuity represents a cooperative structural transition (Figure 6). It appears that PLA has stabilized a population of EYL molecules in a rigid state, preventing their order–disorder transition until 12 °C. The remainder of the EYL molecules undergo a severely perturbed transition, spanning a temperature range from −20 to +15 °C. An unequivocal structural explanation for this complex thermal behavior is difficult at this stage since several separate processes may be involved. It is possible, for example, that PLA preferentially binds acyl chains of a particular length or degree of unsaturation, thus leading to a particular chemical structuring of the "boundary layer" surrounding the protein. In contrast to the DML/PLA system, at very low temperatures the EYL/PLA complex reaches a higher $[I_{\sim 2880}/I_{\sim 2850}]$ than EYL alone, suggesting that on average at these temperatures, the acyl chains of the recombinant possess a more rigid structure than those of EYL alone. We cannot satisfactorily explain this observation at the present time.

In summary, our Raman studies reveal complex effects of a hydrophobic protein on the configurations and thermal properties of phospholipids that are associated with the protein. Previous calorimetric data (Curatolo et al., 1977) and the present studies on DML/PLA recombinants, taken together, indicate that the dimensions of the lipid "boundary layer" existing above the order–disorder transition temperature can be quite large (3–4 phospholipid annuli near the transition temperature of the phospholipid), and that the protein perturbs the lipid structure, albeit to a lesser extent, at higher temperatures. These results are in excellent agreement with the recent theoretical work on lipid-protein interactions by Marcelja (1976). This study predicts extended annuli of "bound" lipid around hydrophobic proteins (4–5 layers) at temperatures near (but above) that of the lipid phase transition and a decrease in the size and order of this "bound" lipid with increasing temperature. Our Raman and calorimetric studies of EYL/PLA recombinants indicate that, once the element of acyl chain heterogeneity is added to the system, the situation becomes more complex, with some of the phospholipid sequestered by the protein into regions which can undergo a cooperative thermal transition at a temperature ~20 °C higher than the normal EYL order–disorder transition.

PLA influences phospholipid bilayers in a manner reminiscent of the action of cholesterol, i.e, it tends to "buffer" membrane fluidity and to reduce the cooperativities of acyl chain transitions. However, it exerts these actions at much lower proportions than cholesterol. (Assuming a monomer molecular weight of 24 000 for PLA (Jolles et al., 1977), the 20:1 (w/w) DML/PLA recombinant corresponds to a molar ratio of ~700:1 DML/PLA). This behavior, predicted for integral membrane proteins by Wallach (1976), has major implications for the interpretations of thermotropic phase transitions in biomembranes (Verma & Wallach, 1976b,c; Verma et al., 1977). The widths of these transitions and the differences observed between the membranes of normal lymphocytes and cells neoplastically transformed by simian virus 40 (Verma et al., 1977) have heretofore been interpreted in terms of membrane cholesterol. The data presented here show that the possible roles of membrane proteins must also be considered.

Acknowledgments

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References

Autilio, L. A., Norton, W. T., & Terry, R. D. (1964) *J. Neurochem. 11,* 17.
Boggs, J. M., Vail, W. J., & Moscarello, M. A. (1976) *Biochim. Biophys. Acta 448,* 517.
Brown, K. G., Peticolas, W. L., & Brown, E. (1973) *Biochem. Biophys. Res. Commun, 54,* 358.
Bulkin, B. J., & Krishnamachari, N. (1972) *J. Am. Chem. Soc. 94,* 1109.
Bunow, M. R., & Levin, I. W. (1977) *Biochim. Biophys. Acta* 487, 388.
Cattell, K. J., Lindop, C. R., Knight, I. G., & Beechey, R. B. (1971) *Biochem. J. 125,* 169.
Chadha, J. S. (1970) *Chem. Phys. Lipids 4,* 104.
Cubero Robles, E., & Van Den Berg, D. (1969) *Biochim. Biophys. Acta 187,* 520.
Curatolo, W., Sakura, J. D., Small, D. M., & Shipley, G. G. (1977) *Biochemistry 16,* 2313.
Folch, J., & Lees, M. (1951) *J. Biol. Chem. 191,* 807.
Folch-Pi, J., & Sakura, J. D. (1976) *Biochim. Biophys. Acta 427,* 410.
Folch-Pi, J., & Stoffyn, P. (1972) *Ann. N.Y. Acad. Sci. 195,* 86.
Gaber, B., & Peticolas, W. L. (1977) *Biochim. Biophys. Acta 465,* 260.
Gagnon, J., Finch, P. R., Wood, D. D., & Moscarello, M. A. (1971) *Biochemistry 10,* 4756.
Gonzalez-Sastre, F. (1970) *J. Neurochem. 17,* 1049.
Hinz, H. J., & Sturtevant, J. M. (1972) *J. Biol. Chem. 247,* 6071.
Jolles, J., Nussbaum, J. L., Shoentgen, F., Mandel, P., and Jolles, P. (1977) *FEBS Lett. 74,* 190.
Jost, P. C., Griffith, O. H., Capaldi, R. A., and Vanderkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 480.
Ladbrooke, B. D., & Chapman, D. (1969) *Chem. Phys. Lipids 3,* 304.
Larsson, K. (1973) *Chem. Phys. Lipids 10,* 165.
Larsson, K., & Rand, R. P. (1973) *Biochim. Biophys. Acta 326,* 245.
Lippert, J. L., & Peticolas, W. L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 1572.
Lis, L. J., Kauffman, J. W., & Shriver, D. F. (1975) *Biochim. Biophys. Acta 406,* 453.
Lis, L. J., Kauffman, J. W., & Shriver, D. F. (1976a) *Biochim. Biophys. Acta 436,* 513.
Lis, L. J., Goheen, S. C., Kauffman, J. W., & Shriver, D. F. (1976b) *Biochim. Biophys. Acta 443,* 331.
Litman, B. J. (1973) *Biochemistry 12,* 2545.
MacLennan, D. H., Yip, C. C., & Iles, G. H. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37,* 469.
Marcelja, S. (1976) *Biochim. Biophys. Acta 455,* 1.
Mendelsohn, R., Sunder, S., & Bernstein, H. J. (1975) *Bio-*

*chim. Biophys. Acta 413,* 329.
Milanovitch, F. P., Yeh, Y., Baskin, R. J., & Harney, R. C. (1976) *Biochim. Biophys. Acta 419,* 243.
Papahadjopoulos, D., Vail, W. J., & Moscarello, M. (1975) *J. Membr. Biol. 22,* 143.
Pinto da Silva, P., & Miller, R. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4046.
Racker, E. (1975) *Biochem. Soc. Trans. 3,* 785.
Racker, E. (1976) *J. Cell. Physiol. 89,* 697.
Schmidt-Ullrich, R., Verma, S. P., & Wallach, D. F. H. (1976) *Biochim. Biophys. Acta 426,* 477.
Snyder, R. G., Hsu, S. L., & Krimm, S. (1978) *Spectrochim. Acta* (in press).
Spiker, R. C., & Levin, I. W. (1975) *Biochim. Biophys. Acta 388,* 361.
Tzagoloff, A., & Meagher, P. (1972) *J. Biol. Chem. 247,* 594.
Verma, S. P., & Wallach, D. F. H. (1975) *Biochim. Biophys. Acta 401,* 168.
Verma, S. P., & Wallach, D. F. H. (1976a) *Biochim. Biophys. Acta 426,* 616.
Verma, S. P., & Wallach, D. F. H. (1976b) *Biochim. Biophys. Acta 436,* 307.
Verma, S. P., & Wallach, D. F. H. (1976c) *Proc. Natl. Acad. Sci. U.S.A. 73,* 3558.
Verma, S. P., & Wallach, D. F. H. (1977a) *Biochim. Biophys. Acta 486,* 217.
Verma, S. P., & Wallach, D. F. H. (1977b) *Biochem. Biophys. Res. Commun. 74,* 473.
Verma, S. P., Wallach, D. F. H., & Schmidt-Ullrich, R. (1975) *Biochim. Biophys. Acta 394,* 633.
Verma, S. P., Schmidt-Ullrich, R., Thompson, W. S., & Wallach, D. F. H. (1977) *Cancer Res. 37,* 3490.
Wallach, D. F. H. (1976) *Med. Hypothesis 2,* 241.
Wallach, D. F. H., & Verma, S. P. (1975) *Biochim. Biophys. Acta 382,* 542.

# Activity and Stability of the Luciferase–Flavin Intermediate†

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ABSTRACT: A luciferase intermediate in the bacterial bioluminescence system, which is formed by reaction of enzyme with reduced flavin mononucleotide ($FMNH_2$) and oxygen, is shown to emit light with added aldehyde under anaerobic conditions. The reaction with oxygen is thus effectively irreversible under the conditions used. The flavin chromophore has an absorption maximum at about 370 nm and the potential activity (bioluminescence yield) in the further reaction of the isolated intermediate with aldehyde is strictly proportional to the amount of this flavin chromophore.

Hastings et al. (1973) utilized low temperature techniques to isolate an enzymatic intermediate in the bacterial bioluminescence reaction formed by the luciferase-catalyzed reaction of $FMNH_2$ with $O_2$ which, based on its spectral characteristics, was postulated to be a luciferase–4a-peroxydihydroflavin complex. Bioluminescence occurs simply upon reacting the intermediate with aldehyde at 25 °C. If the reaction with oxygen is effectively irreversible, then the final steps of the reaction, either via decomposition (without aldehyde) or with luminescence, should occur in the absence of free molecular oxygen. The experiments described here demonstrate that this is the case. This contrasts with the report of Entsch et al. (1976) that the reaction of oxygen with the reduced flavin of *p*-hydroxybenzoate hydroxylase is reversible.

Although the initial isolation (Hastings et al., 1973) of the intermediate was accomplished by chromatography at −30 °C in phosphate-buffered 50% ethylene glycol, the lifetime of the intermediate in aqueous solution at 0 °C is such that it can be isolated and studied at this temperature if the steps are executed promptly. Using such techniques, Murphy et al. (1974) described the isolation of an active intermediate, but one reportedly nearly devoid of flavin. In view of this surprisingly different result and of the profound mechanistic implications if bioluminescence potential is indeed maintained in an aproprotein species, we repeated the isolation under solvent conditions and procedures essentially identical with those of Murphy et al. (1974). Using luciferases purified from two different bacterial species, *Beneckea harveyi* and *Photobacterium fischeri* (Becvar et al., 1976, 1977), we are unable to confirm their report. We find that the intermediates from both luciferases possess a flavin moiety with an absorption spectrum peaking at about 370 nm and an activity (bioluminescence) potential which is strictly proportional to the amount of this flavin intermediate. Upon conversion to product (without aldehyde) we observed clearly defined isosbestic points indicating the existence of only two principal flavin chromophores in the reaction under these conditions.

## Experimental Procedures

Glucose oxidase (type II dry powder), catalase (twice crystallized from beef liver as crystalline suspension), NADH (grade III), DL-dithiothreitol, and FMN were obtained from Sigma. FMN was not purified further since studies revealed that the use of more purified material did not affect the spectral and quantum yield results. FMN concentrations were based on an absorptivity of 12 500 $M^{-1}$ $cm^{-1}$ at 445 nm (Beinert, 1960).

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