teractive Proteins (Jurnak, F. A., & McPherson., A., Eds.) pp 127–170, John Wiley & Sons, New York.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature 282*, 680–686.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., van der Marel, G., van Boom, J. H., & Rich, A. (1981) *Science 211*, 171–176.
Westhoff, E., Dumas, P., & Moras, D. (1985) *J. Mol. Biol. 184*, 119–145.

Hexagonal Phase Forming Propensity Detected in Phospholipid Bilayers with Fluorescent Probes†

Richard M. Epand* and Bryan T.-C. Leon

Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

Received September 5, 1991; Revised Manuscript Received November 1, 1991

ABSTRACT: The fluorescence emission spectrum of N^{ϵ}-dansyl-L-Lys undergoes a marked blue shift when incorporated from aqueous solution into phospholipid bilayers. This shift is greater for membranes composed of dipalmitoleoylphosphatidylcholine than for membranes of dipalmitoleoylphosphatidylethanolamine. With the latter but not the former lipid, the fluorescence emission from N^{ϵ}-dansyl-L-Lys is markedly temperature-dependent. The marked temperature dependence of N^{ϵ}-dansyl-L-Lys fluorescence in bilayers of dipalmitoleoylphosphatidylethanolamine is greatest as the sample is heated close to the bilayer to hexagonal phase transition temperature. The fluorescence emission properties of another probe of membrane surface hydrophobicity, Laurdan, also exhibit marked changes at temperatures just below the bilayer to hexagonal phase transition temperature. At these temperatures, the generalized polarization begins to increase rather than decrease with temperature, and the emission intensity decreases markedly. Such effects are not observed over the same temperature range with phosphatidylcholine. Thus, both dansyl-L-lysine and Laurdan provide probes to measure changes in the physical properties of membrane bilayers which occur when these bilayers are heated close to the temperature required for transition to the hexagonal phase.

The rearrangement of lipid bilayers to morphologically distinct forms has been recognized for many years (Luzzati, 1968). Much attention was given to the possible biological functions of nonbilayer phases (Cullis & deKruijff, 1979). Although some evidence indicates the presence of the nonbilayer inverted hexagonal (H_{II}) phase in biological membranes, the presence of such structures in cell membranes has still not yet been conclusively proven. It is clear that, even if present, the H_{II} phase is not a common or prevalent feature of biological membranes. More recent studies have indicated that the H_{II} itself may not be required for biological function. For example, fusion of phospholipid vesicles does not require the formation of H_{II} phases (Ellens et al., 1989). Nevertheless, bilayer membranes which are more prone to undergo conversion to the H_{II} phase are also more prone to undergo membrane fusion or to promote the activity of certain membrane-bound enzymes (Yeagle, 1989; Hui & Sen, 1989; Epand, 1990). The propensity of a membrane to form the H_{II} phase has been judged by measuring the temperature at which there is a cooperative transition from the bilayer (L_{α}) to the H_{II} phase. However, if shifts in this phase transition temperature are correlated with alterations of the functional properties of bilayer membranes, then the physical properties of the bilayer phase itself must be altered. It is difficult to directly measure changes in the physical properties of the bilayer phase which result from changes in the tendency of each monolayer of the bilayer to curve (Gruner, 1985). This is because these changes in the intrinsic radius of curvature of each monolayer are not expressed as a change in lipid morphology while the membrane is constrained to form a planar bilayer. In the present study, we demonstrate that differences in properties among membrane bilayers can be detected by fluorescence using the probes N^{ϵ}-[5-(dimethylamino)naphthalene-1-sulfonyl]-L-lysine (DNS-Lys)[1] or Laurdan. These properties of the bilayer which affect the fluorescence properties of these probes are correlated with the tendency of the bilayer to form the H_{II} phase.

The fluorescence emission from the dansyl chromophore is known to be very sensitive to the solvent environment. Two types of dansyl derivatives have been used to probe membrane properties. They are dansylated phospholipids (Kimura & Ikegami, 1985; Ohki & Arnold, 1990) and dansylated amino acids. The dansyl group attached to the phospholipid headgroup is relatively fixed in position at the surface of the bilayer, while the dansyl group of dansylated amino acids can intercalate to various depths near the bilayer surface. The fluorescence emission of dansylglycine is particularly sensitive to lipid packing in that it can detect differences between the inner and outer monolayers of small lipid vesicles (Bramhall, 1986). This fluorescent molecule also detects changes in lipid

† Supported by Medical Research Council of Canada Grant MA7654.

[1] Abbreviations: DNS-Lys, N^{ϵ}-[5-(dimethylamino)naphthalene-1-sulfonyl]-L-lysine or dansyl-L-lysine; Laurdan, 6-dodecanoyl-2-(dimethylamino)naphthalene; DiPoPE, L-α-dipalmitoleoylphosphatidylethanolamine; DiPoPC, L-α-dipalmitoleoylphosphatidylcholine; DSC, differential scanning calorimetry; GP, generalized polarization.

0006-2960/92/0431-1550$03.00/0

bilayers as they approach the L_α–$H_\parallel$ transition temperature (our unpublished observations). However, these changes are not completely reversible on recooling. Dansylglycine may be trapped in the membrane when forming a lipid film from organic solvent (which is subsequently hydrated), but it does not readily partition into membranes from the aqueous phase. In contrast, dansyl-L-phenylalanine and DNS-Lys do show reversible changes in fluorescence as one heats and cools lipids near the L_α–$H_\parallel$ transition temperature. The changes with DNS-Lys were somewhat larger, and since it is zwitterionic, its properties should not be very sensitive to membrane charge. We therefore based our current study on the behavior of DNS-Lys in membranes. DNS-Lys has been previously used as a fluorescent probe to study cell membranes. Biological membranes are stained by this substance only if the cells are morbid or have altered membrane properties (Humphries & Lovejoy, 1984). These studies monitored changes in the fluorescence intensity of the probe and not the emission wavelength. Our current study uses model membranes and monitors changes in fluorescence emission properties.

In addition, the fluorescent probe Laurdan also monitors the surface polarity of membranes (Parasassi et al., 1986). The fluorescence emission properties of this probe are particularly sensitive to dipolar relaxation processes caused by the reorientation of water molecules near the membrane surface (Parasassi et al., 1991). The use of this probe, in addition to DNS-Lys, indicates that the alterations in membrane properties that we observe are not probe-dependent.

Materials and Methods

Materials. L-α-Dipalmitoleoylphosphatidylethanolamine (DiPoPE) and L-α-dipalmitoleoylphosphatidylcholine (DiPoPC) were purchased from Avanti Polar Lipids, Inc. (Pelham, AL); DNS-Lys and Laurdan were from Molecular Probes, Inc. (Eugene, OR). The above were shown to be pure by TLC and were used without further purification.

Sample Preparation. Typically, 10 mg of DiPoPE was dissolved in chloroform/methanol (2/1, v/v) together with known amounts of DNS-Lys or Laurdan. The solutions were dried down to a lipid film under a stream of nitrogen gas. The films were then put under high vacuum for 2 h to eliminate traces of solvent. To each film was added 2 mL of Pipes buffer made up of 20 mM Pipes, 1 mM EDTA, 150 mM NaCl, and 0.02% NaN_3 with a final pH of 7.4. The samples were heated to 45 °C and vigorously vortexed.

Differential Scanning Calorimetry (DSC). After the lipid suspension was degassed under vacuum, the Microcal MC-2 liquid-sample cell was loaded. An equal volume of Pipes buffer was used in the reference cell. Thermograms were obtained at a heating scan rate of 45 °C/h. Microcal software was used to analyze the data.

Spectrofluorometry. Fluorescence experiments were performed on a Perkin-Elmer MPF-44 fluorescence spectrophotometer. The excitation wavelength was 340 nm (Laurdan) or 350 nm (DNS-Lys), and the emission spectrum was measured from approximately 390 to 580 nm with a scan rate of 60 nm/min. The excitation and emission slit widths were 8 nm. Samples were placed in a 5-mm-diameter cuvette, and magnetic stirring was used to provide a homogeneous suspension throughout the measurements. The temperature was controlled using a circulating water bath. Sample temperature was monitored with a thermister probe in the sample cuvette.

Results

The fluorescence emission of the dansyl group may reflect the hydrophobicity of its environment. Two assumptions are implicit in making this correlation. One is that solvent reorientation is rapid compared with the rate of decay of the excited singlet state. The second assumption is that the environment can be described by a single dielectric constant. While these assumptions are likely to be correct for DNS-Lys in solution, they may not be accurate for the probe in the condensed phase of a membrane where there is restricted motion and nonuniform microenvironments [see, for example, Dodiuk et al. (1979)]. Nevertheless, to give an estimation of the nature of the environment of the dansyl group in membranes and to show relative changes with temperature or with the chemical constituents of the membrane, we have expressed the results in terms of the apparent dielectric constant of the environment. In order to do, this we have measured the fluorescence spectrum of DNS-Lys in a series of dioxane/water mixtures of known [given in Bramhall (1986)] dielectric constant (Figure 1). Similar results have been reported for DNS-Gly (Bramhall, 1986). We have used our smoothed calibration curve (Figure 1) to convert emission wavelength to an estimate of the dielectric constant.

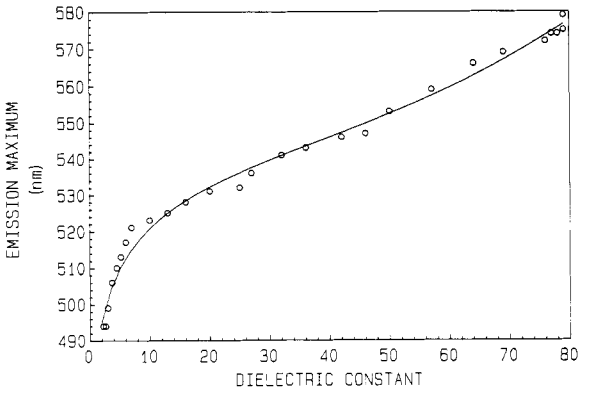

FIGURE 1: Dependence of DNS-Lys emission on solvent dielectric constant. The emission maximum of DNS-Lys was measured at room temperature from a 1 μM solution of DNS-Lys in a series of water/dioxane mixtures of known dielectric constant. The emission wavelength was insensitive to changes in temperature over the range 10–30 °C.

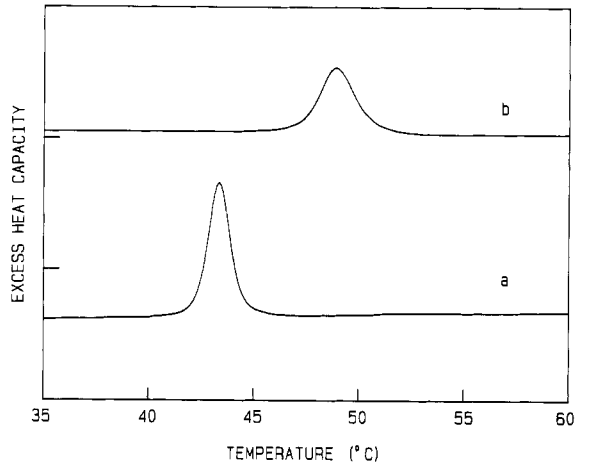

FIGURE 2: DSC scan of DiPoPE (5 mg/mL) in the absence (a) or the presence of 2 mol % DNS-Lys (b). Heating scan rate 39 K/h. Each tick mark on the vertical axis represents 50 cal K^{-1} (mol of DiPoPE)$^{-1}$.

We studied the properties of DNS-Lys in membranes of DiPoPE. This phosphatidylethanolamine has a bilayer–hexagonal transition temperature at 43.5 °C (Silvius et al., 1985; and this work). In the presence of 2 mol % DNS-Lys, this transition temperature is shifted to 48.9 °C (Figure 2). In the presence of lipid, most of the DNS-Lys spontaneously

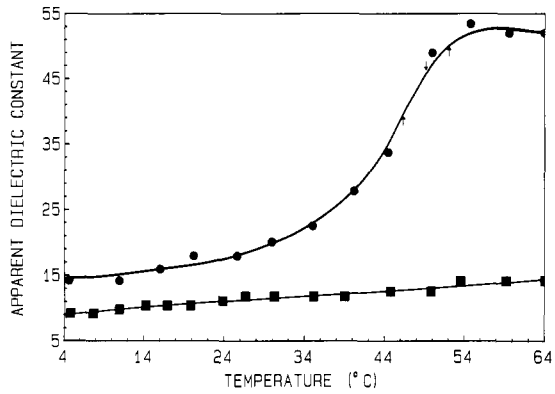

FIGURE 3: Temperature dependence of the solvent environment of DNS-Lys in membranes. The apparent dielectric constant is calculated from the emission maximum using the calibration curve of Figure 1. The mole fraction of DNS-Lys is 0.018. Multilamellar vesicles (4 mg/mL) are composed of DiPoPC (■) or DiPoPE (●). The arrow pointing down marks the temperature of the L_α–H_{II} transition of DiPoPE as measured by DSC. Upward arrows define the upper and lower temperatures of the mixed phase region.

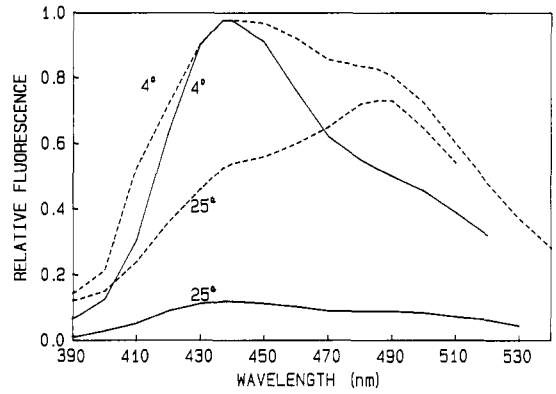

FIGURE 4: Examples of fluorescence emission spectra of Laurdan in DiPoPE (solid curves) or DiPoPC (dashed curves). Temperatures that the spectra were obtained are given in the figures (degrees celsius). The phospholipid concentration is 1.5 mg/mL with 0.0015 mole fraction of Laurdan. Emission intensities of DiPoPC and DiPoPE were arbitrarily normalized to the same value at 440 nm for the 4 °C spectra. Relative intensities of the spectra of 4 and 25 °C are accurately given.

partitions from the aqueous to the membrane phase. This is shown by the fact that the fluorescence emission spectrum of DNS-Lys in the presence of DiPoPE at low temperature shows a single emission peak below 530 nm. In buffer, the emission of DNS-Lys is above 550 nm. There is no shoulder at higher wavelengths for the emission spectrum of DNS-Lys in the presence of lipid, indicating that there is no aqueous form of DNS-Lys which exchanges with the membrane at a rate slower than the rate of fluorescence decay. The widths of the emission spectra are the same in buffer and in lipid.

The temperature dependence of the DNS-Lys emission indicates that the environment of this probe in DiPoPE membranes changes rapidly above about 30 °C, suggesting exposure of the probe to a more polar environment with increasing temperature (Figure 3). This temperature sensitivity is not observed for DNS-Lys in DiPoPC membranes where it experiences an apparently more hydrophobic environment which is relatively insensitive to temperature (Figure 3). The apparent dielectric constant was calculated using the calibration curve shown in Figure 1. This calibration curve was shown to be insensitive to temperature over the range 10–30 °C. We utilized this same calibration curve to calculate approximate apparent dielectric constants up to 64 °C. The fact that the fluorescence emission of DNS-Lys in DiPoPC does not change much up to this high temperature supports our use of the same calibration curve to higher temperatures. There is no calibration curve for the probe in free solution which would accurately represent the anisotropic environment of the probe in a membrane. The main observation is the relative sensitivity to temperature change with DiPoPE and not with DiPoPC, rather than the actual values of the apparent dielectric constant. Despite the large shift of the fluorescence emission of DNS-Lys between membrane and aqueous environments, the absorption spectrum of this probe retains a band at about 330 nm in both environments. In the case of DiPoPE, the change in the environment of DNS-Lys is virtually complete by 48.9 °C, the bilayer to hexagonal phase transition temperature of DiPoPE in the presence of 2 mol % DNS-Lys. In fact, a large fraction of the shift in fluorescence emission of DNS-Lys occurs even below the temperature of onset of the phase transition (indicated by arrows in Figure 3). The behavior of DNS-Lys in DiPoPE is insensitive to the mole fraction of DNS-Lys used between 0.2 and 2 mol %.

In contrast to DNS-Lys which exhibits a single peak in its fluorescence emission spectrum, the fluorescent probe Laurdan

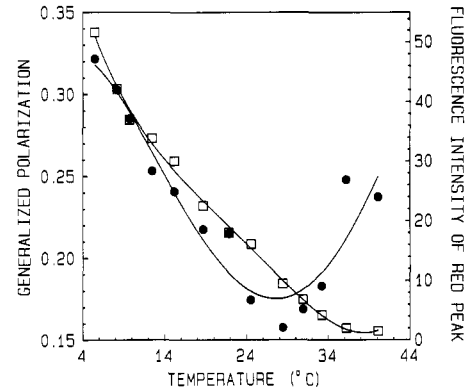

FIGURE 5: Temperature dependence of the solvent environment of Laurdan in DiPoPE. Phospholipid concentration 1.5 mg/mL with 0.0015 mole fraction of Laurdan. Generalized polarization (●) and the intensity of the high-wavelength emission peak (□).

exhibits two maxima in its emission spectrum. The emission spectrum of Laurdan in membranes has maxima at about 440 and 490 nm (Parasassi et al., 1991; Figure 4). The relative intensity of these two peaks is dependent on the conditions. The relationship of these intensities can be expressed as the generalized polarization (GP):

$$GP = \frac{I_B - I_R}{I_B + I_R}$$

where I_B and I_R are the emission intensities at the blue and red edges of the emission spectrum, respectively. The wavelengths taken for measurement of I_B and I_R corresponded to the wavelengths of the two emission bands at about 440 and 490 nm, as previously described (Parasassi et al., 1991). The intensity of the high-wavelength peak was also recorded. With DiPoPE, the GP decreases with temperature between 5 and 28 °C (Figure 5). Similar behavior has been observed with other lipids in the liquid-crystalline state (Parassi et al., 1991). However, above about 28 °C, the GP begins to increase, and the intensity of the fluorescence becomes very small. The spectrum of Laurdan at these higher temperatures resembles that of Laurdan in buffer without lipid. The marked changes in Laurdan fluorescence occur at temperatures below the onset of the bilayer to hexagonal phase transition as measured by DSC (at the low mole fractions of Laurdan used, the DSC curve resembles that of the pure DiPoPE shown in Figure 2,

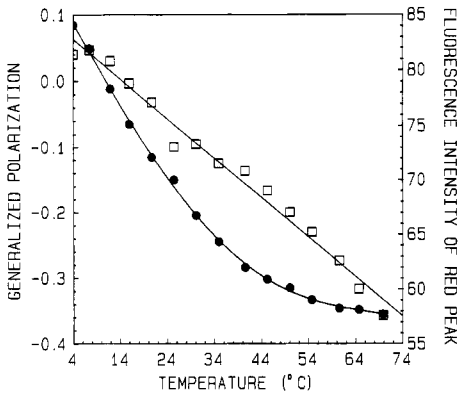

FIGURE 6: Temperature dependence of the solvent environment of Laurdan in DiPoPC. Phospholipid concentration 1.5 mg/mL with 0.0015 mole fraction of Laurdan. Generalized polarization (●) and the intensity of the high-wavelength emission peak (□).

curve a). Similar changes in fluorescence properties were obtained with mole fractions of Laurdan between 0.001 and 0.003.

Laurdan exhibited very different behavior with DiPoPC (Figure 6). There is a continuous decrease in the GP which becomes somewhat less at higher temperatures, but it never increases with temperature. The decrease in fluorescence intensity with temperature for DiPoPC was relatively modest compared with the behavior of DiPoPE. Note that the temperature range for which the Laurdan fluorescence could be studied was greater for DiPoPC than for DiPoPE and the intensity scale in Figure 6 does not go to zero.

Discussion

Substances that raise the L_α–$H_\parallel$ transition temperature have been shown to affect membrane properties at low mole fractions. For example, both cholesterol sulfate (Cheetham et al., 1990) and carbobenzoxy-D-Phe-L-PheGly (Kelsey et al., 1990) inhibit viral fusion to phospholipid vesicles. Cholesterylphosphoryldimethylethanolamine is an example of a bilayer-stabilizing amphiphile that inhibits protein kinase C (Epand et al., 1989). These effects are observed under conditions where there is little likelihood of hexagonal phase formation. Yet the ability of amphiphiles to inhibit membrane fusion or to inhibit protein kinase C activity is often correlated with the ability of these amphiphiles to raise the L_α–$H_\parallel$ transition temperature. This suggests that the propensity of a membrane to form the hexagonal phase is correlated with some change in a physical property of the bilayer.

A change in a property of the bilayer which occurs as it approaches the L_α–$H_\parallel$ transition temperature is detected by DNS-Lys (Figure 3). The marked change in DNS-Lys fluorescence occurs in a temperature range from 35 to 45 °C where the membrane remains in the bilayer phase. Thus, DNS-Lys is able to detect the increased hexagonal phase forming propensity of the bilayer membrane. The effect is particularly marked in a temperature range just below the transition to the hexagonal phase although the fluorescent probe is not particularly sensitive to the formation of the hexagonal phase itself. The marked temperature dependence of the fluorescence emission of DNS-Lys is not observed in phosphatidylcholine bilayers which have little propensity to form the hexagonal phase (Figure 3).

A schematic model to interpret these results can be proposed if one assumes that the fluorescence emission is monitoring the dielectric constant of the environment. This has not been proven. We suggest that in a stable bilayer, such as that of DiPoPC, the dansyl group shows a large blue shift from its

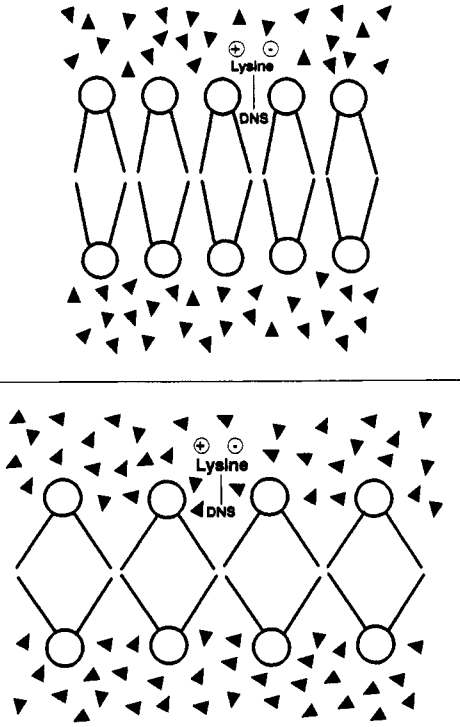

FIGURE 7: Schematic diagram of the influence of hexagonal phase propensity on the fluorescence of DNS-Lys. The top figure represents a stable bilayer in which the lipid headgroups form a barrier to water (black triangles) penetration. The dansyl (DNS) group of DNS-Lys is sequestered in a relatively hydrophobic environment. The bottom figure represents a bilayer at a temperature just below the temperature at which it is converted to the hexagonal phase. Here water is able to penetrate deeper into the membrane and thereby make the solvent environment of the dansyl group more polar.

emission in water, indicative of its sequestration within the membrane (Figure 7). In contrast, in DiPoPE, the dansyl group of DNS-Lys is in a slightly more aqueous-like environment at low temperatures but enters into a particularly hydrophilic environment as the temperature of the L_α–$H_\parallel$ transition is approached (Figure 7). Factors which may affect the emission maximum of DNS-Lys in membranes include the depth of penetration of water into the bilayer and the depth of membrane insertion of the dansyl group. Both of these effects are illustrated in Figure 7. With increased separation of the phospholipid headgroups, water can penetrate more deeply into the membrane, and the dansyl group will be less sequestered within the membrane.

Laurdan is also an environment-sensitive probe. It differs from DNS-Lys in being uncharged rather than zwitterionic, and in having a lauryl group for membrane-anchoring. The temperature dependence of the GP is small for gel-state lipid (Parasassi et al., 1991) but is greater for liquid-crystal-state lipid (Parasassi et al., 1991; Figures 4 and 5 of this work). This is in accord with a greater rate of solvent relaxation at higher temperatures in liquid-crystalline membranes, leading to a stabilization of the excited state. This causes a red shift in the fluorescence emission. The GP of DiPoPE (Figure 5) is higher than that of DiPoPC (Figure 6) as expected for the more rigid, H-bonded, headgroup structure of DiPoPE. Above 34 °C, the Laurdan fluorescence becomes much weaker, and the GP begins to increase. The values of GP at higher temperatures are less accurate because of the low intensity of fluorescence. The changes may be caused by expulsion of the probe from the membrane or by marked alterations in the physical properties of the membrane surface. Regardless of the origin of these spectral changes, the fact that they are

occurring well below the bilayer to hexagonal phase transition temperature indicates that Laurdan can also detect changes in membrane bilayer properties which are correlated with the tendency to form the hexagonal phase. The fact that the chemical and physical properties of Laurdan are different from DNS-Lys, yet both probes detect these changes in bilayer properties, gives further support that these probes are monitoring intrinsic properties of the membrane.

The results of the present paper demonstrate that bilayers with a high propensity for forming the hexagonal phase have markedly altered physical properties. Changes in the stability, hydrophobicity, and solvation of the bilayer surface may be responsible for the changes detected by the fluorescent probes. These alterations in membrane surface properties may also cause membrane functional changes which do not specifically require the formation of nonbilayer phase intermediates.

Registry No. DNS-Lys, 28217-24-5; DiPoPC, 56815-99-7; DiPoPE, 61599-23-3; Laurdan, 74515-25-6.

References

Bramhall, J. (1986) *Biochemistry 25*, 3479-3486.

Cheetham, J. J., Epand, R. M., Andrews, M., & Flanagan, T. D. (1990) *J. Biol. Chem. 265*, 12404-12409.

Cullis, P. R., & deKruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399-420.

Dodiuk, H., Kanety, H., & Kosower, E. M. (1979) *J. Phys. Chem. 83*, 515-521.

Ellens, H., Siegel, D. P., Alford, D., Yeagle, P., Boni, L., Lis, L., Quinn, P. J., & Bentz, J. (1989) *Biochemistry 28*, 3692-3703.

Epand, R. M. (1990) *Biochem. Chem. Biol. 68*, 17-23.

Epand, R. M., Stafford, A. R., Bottega, R., & Ball, E. H. (1989) *Biosci. Rep. 9*, 315-328.

Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665-3669.

Hui, S.-W., & Sen, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5825-5829.

Kelsey, D. R., Flanagan, T. D., Young, J., & Yeagle, P. L. (1990) *J. Biol. Chem. 265*, 12178-12183.

Kimura, Y., & Ikegami, A. (1985) *J. Membr. Biol. 85*, 225-231.

Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.), Vol. 1, pp 71-123, Academic Press, London and New York.

Ohki, S., & Arnold, K. (1990) *J. Membr. Biol. 114*, 195-203.

Parasassi, T., Conti, F., & Gratton, E. (1986) *Cell. Mol. Biol. 32*, 103-108.

Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M., & Gratton, E. (1991) *Biophys. J. 60*, 179-189.

Yeagle, P. L. (1989) *FASEB J. 3*, 1833-1842.

Nucleosome Spacing Is Compressed in Active Chromatin Domains of Chick Erythroid Cells†

Bryant Villeponteau,‡ James Brawley,§,‖ and Harold G. Martinson*,‖

Department of Biological Chemistry and Institute of Gerontology, The University of Michigan, Ann Arbor, Michigan 48109-2007, and Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024

Received May 29, 1991

ABSTRACT: We have cleaved the chromatin of embryonic and adult chicken erythroid cells using a novel nuclease that is capable of resolving clearly the nucleosomes of active chromatin. We found that in active chromatin, nucleosomes are spaced up to 40 base pairs closer together than in inactive chromatin. This was true for both "housekeeping" and "luxury" genes and was observed whether the digestion was carried out on isolated nuclei in vitro or by activating the endogenous nuclease in vivo. The close spacing extended several kilobases into flanking chromatin, indicating that this is a domain property of active chromatin, not just a characteristic of regions disrupted by transcription. A simple interpretation of our results is that the nucleosomes of active chromatin are mobile in vivo and, not being constrained by linker histones, freely move closer together.

Shortly after the discovery of the nucleosome as the fundamental subunit of chromatin (Kornberg, 1974), it was recognized that the average nucleosome periodicity varies widely from one organism and tissue to another (Eissenberg et al., 1985). The spacing between nucleosomes, averaged over the whole genome, is correlated with the overall degree of transcriptional activity of the cell. During the course of development, increasing transcriptional activity is correlated with decreasing internucleosomal repeat distance (Brown & Sutcliffe, 1987) whereas decreases in transcriptional activity are correlated with increases in the internucleosomal repeat distance (Chambers et al., 1983; Weintraub, 1978). For example, chicken erythroid cells, the subject of the present report, exhibit a steady increase in average nucleosome spacing throughout the genome during maturation (Weintraub, 1978). Focusing on vertebrate RNA polymerase II for our present discussion, several studies of specific genes also provide evidence consistent

† This work was supported by Grant GM 35750 from the National Institutes of Health and by Grant CA-385 from the American Cancer Society.

‡ The University of Michigan.

§ Present address: Virginia Mason Research Center, 1000 Seneca St., Seattle, WA 98101.

‖ University of California, Los Angeles.

0006-2960/92/0431-1554$03.00/0