Hydrocarbon Chain Trans–Gauche Isomerization in Phospholipid Bilayer Gel Assemblies†

Nehama Yellin and Ira W. Levin*

ABSTRACT: The vibrational Raman spectra of dimyristoyl (DMPC)-, dipalmitoyl (DPPC)-, and distearoylphosphatidylcholine (DSPC)–water bilayer systems were used to probe lipid hydrocarbon chain trans–gauche isomerization dynamics below the gel–liquid crystalline phase transition temperature. In addition to the 1090–1085 cm^{-1} vibrational transitions, which appear with increasing temperatures and are characteristic of gauche conformers within the acyl chains, a new feature arises in all three bilayer systems at ~1122 cm^{-1}. This carbon–carbon stretching mode is associated with the formation of a gauche bond rotation of the terminal methyl group oriented toward the center of the bilayer. Estimates of the enthalpy differences (ΔH) between hydrocarbon chains in an all-trans conformation and chain configurations containing gauche forms may be made from peak height intensities of vibrational features associated with the appropriate rotational isomers. For the DMPC–H_2O, DPPC–H_2O, and DSPC–H_2O assemblies, the Raman data yield enthalpy differences of 2.9 ± 0.6, 3.4 ± 0.5, and 9.9 ± 1.2 kcal/mol, respectively. These values are interpreted to reflect approximately two gauche bonds per lipid molecule for the DMPC–H_2O and the DPPC–H_2O gels and six gauche bonds per molecule for the DSPC–H_2O gels.

As a phospholipid bilayer assembly undergoes a phase transition from the gel (or crystalline) form to the liquid crystalline state, the hydrocarbon chains experience both interchain and intrachain disorder leading to a model in which the bonds nearest the terminal methyl groups in the bilayer interior manifest relatively unrestricted conformational mobility (see, for example, Lee, 1975; McCammon and Deutch, 1975; Schindler and Seelig, 1975; Jacobs et al., 1975; Jackson, 1976). The hydrocarbon chains in the low-temperature gel, or crystalline, modification are generally considered to reflect either all-trans arrangements or a packing situation in which the acyl chains are in near-trans conformations (Rothman, 1973; Levine, 1972; Traüble, 1972; Lippert and Peticolas, 1971, 1972). In an effort to determine more fully the flexibility, or intramolecular disorder, of the hydrocarbon chains at temperatures below the transition temperature, we examine in the present study the vibrational Raman spectroscopic behavior of several phospholipid–water bilayer systems in the carbon–carbon stretching mode spectral region. Specifically, the temperature dependence of bilayer systems of dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine (DMPC, dppc, and DSPC, respectively)[1] was monitored from slightly above liquid nitrogen temperatures (~−180 °C) to a point slightly below the pretransition, or lower transition, temperature. The enthalpy difference between rotational isomers for each system was estimated from the Raman spectral data and was interpreted in terms of the number of gauche rotamers formed.

Experimental Section

The phospholipids 1,2-dimyristoyl-DL-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-DL-phosphatidylcholine (DPPC), and 1,2-distearoyl-DL-phosphatidylcholine (DSPC) were obtained from Sigma Chemical Co. Since no major spectral contaminants were observed, the samples were used without further purification. Samples of phospholipid–water multilayers were prepared by weighing the appropriate amounts of phospholipid and water (the total weight varied between 150 and 200 mg). The two components were mixed thoroughly and heated for several seconds to about (T_{m2} + 10) °C, where T_{m2} represents the primary gel to liquid crystalline phase transition temperature of the system.[2] The mixing and heating procedure was repeated several times (two to five) until a homogeneous gel was ultimately established. The gels contained 31–36% H_2O.

The phosphatidylcholine samples were recorded from either a variable-temperature vacuum cell (Levin, 1969) or a suitably thermostated capillary. Sample temperatures, varied between −180 and $T < T_{m1}$, where T_{m1} represents the lower transition or pretransition temperature,[2] were monitored by a copper–constantan thermocouple inserted within the gel as close as possible to the laser beam transit. Temperature control was maintained within ±2 °C. An accuracy of ±3 °C was estimated for each temperature measurement.

Spectra were recorded with a Cary 81 Raman spectrophotometer equipped with a modified external optical system and a Coherent Radiation Model 52 argon ion laser source. Typical laser powers of 400 mW of 514.5-nm radiation were employed. The spectral resolution was 3.4 cm^{-1}. Frequencies, calibrated for each scan with atomic argon lines, are reported to within ±2 cm^{-1}.

Since curve resolution becomes quite complicated for the spectra of these phospholipid systems at higher temperatures, intensity ratios for Raman transitions are reported as peak height ratios to maintain consistent comparisons between molecular systems. The band intensities of the carbon–carbon (C–C) gauche stretching modes in the spectral range 1090-

† From the Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received July 20, 1976.*

[1] Abbreviations used: DMPC, DPPC, and DSPC, dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

[2] Transition temperatures and enthalpy changes for dilute solutions of phospholipids, determined by Hinz and Sturtevant (1972), are as shown in Table III.

TABLE I: Frequencies of Raman Transitions as a Function of Temperature in the C-C Stretching Region for the Gel Forms of DMPC, DPPC, and DSPC Phospholipid-Water Dispersions.

Phospholipid Multilayer	T (°C)	Frequency (cm^{-1})	T (°C)	Frequency (cm^{-1})	Assignment
DMPC	−176	1062	+15	1062	Trans form C-C stretch
DPPC	−180	1062	+28	1062	
DSPC	−180	1062	+45	1062	
DMPC	−176		+15	1090-1085	Gauche form C-C stretch
DPPC	−180		+28	1090-1085	
DSPC	−180		+45	1090-1085	
DMPC	−176	1092	+15	1088	Trans form C-C stretch
DPPC	−180	1101.5	+28	1097	
DSPC	−180	1103	+45	1100	
DMPC	−176		+15	1122	Gauche form C-C stretch
DPPC	−180		+28	1123	
DSPC	−180		+45	1122	
DMPC	−176	1129	+15	1127	Trans form C-C stretch
DPPC	−180	1130.5	+28	1127	
DSPC	−180	1132	+45	1127.5	

1085 cm^{-1} were corrected for each scan by subtracting the background recorded for the all-trans system at −180 °C. We assume that the thermal effect on the background, which includes the wings of other bands and the underlying phosphate stretching mode, can be neglected compared with the significant change in intensity arising in the region of the C-C gauche stretching vibrations. The band intensities of the C-C all-trans modes at 1062 and 1130 cm^{-1} were corrected in the conventional manner for the slight overlapping of neighboring bands. The 1130 cm^{-1} transition peak intensity, however, was not corrected for the appearance of the relatively weak 1122 cm^{-1} shoulder.

Results

The hydrocarbon skeletal C-C stretching region, which extends for our purposes from 1000 to 1150 cm^{-1}, provides the most convenient spectral region for observing changes in conformation of the acyl chains. Vibrational assignments for polycrystalline DPPC, which have been discussed earlier (Spiker and Levin, 1975), are readily transferred to the phospholipid multilayer systems. The three phospholipid dispersions examined, 65% DMPC-35% H_2O, 69% DPPC-31% H_2O, and 64% DSPC-36% H_2O, differ only in their hydrocarbon chain length of 14, 16, and 18 carbon atoms, respectively. Basically, each phospholipid system exhibits the same spectral pattern with slight differences in relative intensities and absolute frequencies. Table I summarizes the C-C skeletal frequency shifts for the three systems as the temperature of the gel increases from about −180 °C to slightly below the lower transition point. The changes in the spectrum between these temperature extremes are displayed in Figure 1 for DSPC multilayers. Although the spectral frequency and intensity changes are similar for the three lipids, the rates of change are characteristic for each system in the temperature range $-180\ °C < T < T_{m1}$. While the 1062 cm^{-1} band frequency neither shifts with chain length nor with temperature, the 1132-1129 and 1103-1092 cm^{-1} bands are found to be frequency sensitive to both chain length and temperature, as summarized in Table I. The shift in frequency is toward lower values with increasing temperature and decreasing chain length.

At −180 °C no C-C gauche transitions are observed; all the major observed transitions in the 1000-1150 cm^{-1} region are attributed to C-C all-trans stretching modes. [The phosphate symmetric and C-O stretching modes are quite weak and probably lie under the ~1090 and ~1050 cm^{-1} areas, respectively (Spiker and Levin, 1975)]. Although the C-C trans stretching modes lose intensity with increasing temperature, the thermal behavior of the 1103-1092 cm^{-1} modes is uncertain as they are severely overlapped by the C-C gauche modes which simultaneously appear at higher temperatures in the 1090-1085 cm^{-1} region. The 1122 cm^{-1} transition, which is absent at −180 °C and only appears on increasing the temperature, is also assigned as a C-C stretching mode characteristic of a gauche conformation. The 1122 cm^{-1} feature, which has not been previously reported, is moderately weak at the highest temperature studied; hence, the frequency determination is of relatively low accuracy, and no firm conclusions can be made with respect to a frequency shift either with temperature or with chain length. Figure 2 shows the appearance of the 1122 cm^{-1} feature in the DMPC, DPPC, and DSPC systems as the temperatures increase from ~−180 °C to their respective values slightly below T_{m1}. The increase in intensity of the 1090-1085 cm^{-1} area, which contains overlapping spectral transitions, is reflected in a frequency shift of the 1092 cm^{-1} maximum to 1088 cm^{-1} for the DMPC-H_2O system and of the 1101.5 cm^{-1} band maximum to 1097 cm^{-1} for the DPPC-H_2O system. Only for the DSPC-H_2O

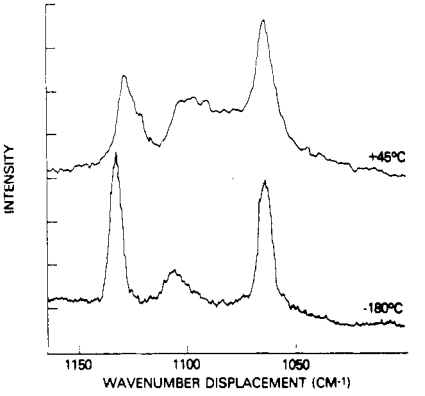

FIGURE 1: Raman spectra of a DSPC-water gel in the hydrocarbon C-C skeletal region at −180 and 45°C.

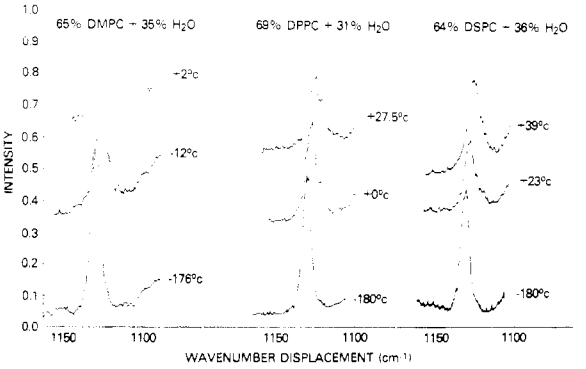

FIGURE 2: Raman spectra of DMPC-, DPPC-, and DSPC-water gels showing (a) the frequency and intensity changes of the 1130 cm^{-1} C-C all-trans stretching mode as a function of temperature and (b) the appearance of the 1122 cm^{-1} C-C gauche feature as the temperature increases from ~−180 °C.

system are two relatively distinct, although overlapping, transitions, at approximately 1100 and 1085 cm^{-1}, observed. (Refer to Table I and Figure 1.)

For the gel state the enthalpy difference $\Delta H_{T<T_{m1}}$ between the hydrocarbon chains in an all-trans conformation and a configuration containing rotational isomers may be estimated from the Raman data once the appropriate spectral identifications have been made. The relationship between the Raman peak intensities I and ΔH is expressed by the integrated form of the van't Hoff equation

$$\ln(I_{\text{gauche}}/I_{\text{trans}}) = -\frac{\Delta H_{T<Tm1}}{RT} + c$$

where I_{trans} and I_{gauche} are proportional to the all-trans and rotameric conformers, respectively. R and T represent the gas constant and temperature, respectively. Although integrated Raman intensities should probably be used in the expression for computing ΔH, the complexity of the systems suggests our substitution of peak height values. We note, in support for this approach, that early infrared studies on the rotational isomers of 1,2-dichloroethane detected no significant differences in enthalpy values for the use of either peak or integrated intensities (Bernstein, 1949). In the expression I_{gauche} is represented by I_{1088} for DMPC, for example, while I_{trans} is represented by either I_{1129} or I_{1062}. Plots of either $\ln(I_{1088}/I_{1129})$ or $\ln(I_{1088}/I_{1062})$ as a function of $1/T$ yield slopes related to the enthalpy differences of the trans-gauche isomerization. The temperature ranges for the plots extend from the point at which the gauche structures first appear, approximately −40 °C for DMPC-H_2O and DPPC-H_2O and about 5 °C for DSPC-H_2O (Yellin and Levin, 1976), to a final temperature which is slightly lower than T_{m1}. Both types of plots give equivalent ΔH values for the phospholipid-water dispersions within their respective range of errors, although the slopes based upon the 1088 and 1129 cm^{-1} spectral transitions appear more precise. The decrease in precision of the $\ln(I_{1088}/I_{1062})$ plots perhaps results from an overlapping of the C-C stretching mode with the C-O stretching vibration at 1052 cm^{-1}. The agreement in ΔH values between the two plots based upon different all-trans modes suggests that the approximations involved in using peak height intensities, rather than the areas of curve resolved spectra, are probably valid and that consistent enthalpy values for these phospholipid systems may be determined from Raman data. Figure 3 shows the analysis for DMPC, DPPC, and DSPC using the I_{1088}/I_{1129} ratios. Values for $\Delta H_{T<T_{m1}}$ from least-squares fits of both types of plots are summarized

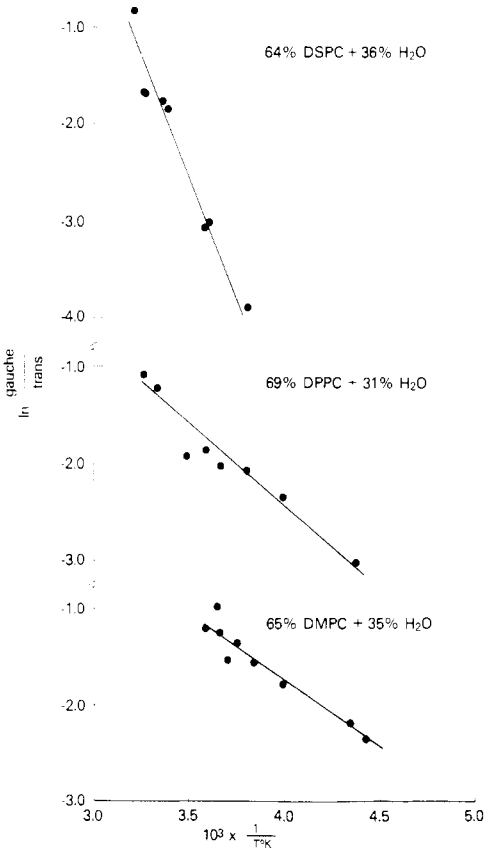

FIGURE 3: Temperature dependence plots of $\ln(I_{\text{gauche}}/I_{\text{trans}})$ for hydrocarbon chain trans-gauche isomerization for DMPC-, DPPC-, and DSPC-water gels. I_{gauche} and I_{trans} represent the peak height intensities of the 1088 and 1129 cm^{-1} Raman transitions, respectively.

in Table II. The dispersions in ΔH were estimated by accepting a value three times that of the least-squares standard deviation. The ΔH values for the DMPC-H_2O and DPPC-H_2O systems are quite close to one another, while the DSPC-H_2O system results in a significantly higher value.

Discussion

The 1000-1150 cm^{-1} C-C skeletal stretching region reflects the emergence of hydrocarbon chain gauche structures in the gel state as the phospholipid bilayers increase in temperature from about −180 °C to temperatures slightly below the crystalline-liquid crystalline phase transition. As expected, the three vibrational transitions characteristic of an all-trans acyl chain, 1062, 1092-1103, and 1129-1132 cm^{-1}, exhibit the same frequency correlation with chain length as that found in a homologous series of saturated fatty acids (Lippert and Peticolas, 1972). As we noted earlier, the 1129-1132-cm^{-1} transition is also temperature sensitive. A sensitivity to temperature in the 1103-1092 cm^{-1} feature is not clearly established as these transitions become severely overlapped by the C-C stretching modes characteristic of gauche conformers which appear in the 1090-1085 cm^{-1} region at higher temperatures. (The apparent frequency shift in this region may also be interpreted as C-C transitions arising from a strictly all-trans fragment, but from a chain shortened form; that is, the gauche rotation effectively decouples a trans fragment from the remainder of the chain.)

Since normal coordinate calculations indicate that the 1129-1132 cm^{-1} C-C stretching mode is coupled to the chain

TABLE II: Enthalpy Values for the Difference in State between the Hydrocarbon All-trans and Gauche Isomeric Forms of Phospholipid–Water Gels.

Phospholipid System	$\Delta H_{T<T_{m1}}$ (kcal/mol) (1130 cm^{-1})[a]	$\Delta H_{T<T_{m1}}$ (kcal/mol) (1062 cm^{-1})[b]	n_g, $T < T_{m1}$[c]	n_g, $T > T_{m2}$[d]
65% DMPC–35% H_2O	2.9 ± 0.6	3.3 ± 0.6	~2	~7
69% DPPC–31% H_2O	3.4 ± 0.4	3.1 ± 1.9	~2	~8–9
64% DSPC–36% H_2O	9.9 ± 1.2	10.0 ± 3.2	~6	~13–14

[a] Enthalpy plot based upon $I_{1130cm^{-1}} \equiv I_{trans}$. [b] Enthalpy plot based upon $I_{1062cm^{-1}} \equiv I_{trans}$. [c] n_g represents an estimate for the number of gauche bonds per phospholipid molecule formed at $T < T_{m1}$, using a value of ~1.7 kcal/mol for the enthalpy difference between the all-trans and gauche conformations about a carbon–carbon bond (see text). [d] n_g represents an approximate upper limit to the number of gauche bonds formed at $T > T_{m2}$ (see text).

terminal methyl rocking mode (Snyder, 1967; Schachtschneider and Snyder, 1963), frequency shifts for this C–C stretching vibration would occur, as observed, as the methyl motions become less restrictive for an increase in temperature. The 1062 cm^{-1} feature is a relatively pure stretching mode and, although the intensity of this transition changes with temperature, no significant frequency shifts arise. For gel systems then, the 1062 cm^{-1} feature provides an internal frequency calibration band.

Changes in the peak heights of the all-trans modes, reported at 1124 and 1064 cm^{-1}, have been previously observed as a function of temperature in the gel form, but for only the dioleoyllecithin multilayer system (Lippert and Peticolas, 1972). For this molecule a cis double bond occurs at the 9 position of the hydrocarbon chain. Since the double bond disorders the arrangement of the hydrocarbon chains in the gel, the intensity differences in the gel were attributed, in general, to increased molecular motions (Lippert and Peticolas, 1972).

The distinct feature at 1122 cm^{-1}, which is also associated with the formation of gauche conformations, appears as a shoulder for $T < T_{m1}$ and would lie under the 1123 cm^{-1} peak in the liquid crystalline phase for $T \geq T_{m2}$. Again, two possibilities arise for the interpretation of this feature: (a) the 1122 cm^{-1} shoulder is a C–C stretching mode characteristic of a short trans chain segment or (b) the 1122 cm^{-1} transition is characteristic of the entire chain whose terminal methyl group is involved in a gauche conformation. The appearance of an 1123 cm^{-1} liquid crystalline mode probably results from the formation of a number of trans chain segments separated by gauche bond rotations, analogous to that which occurs in polymethylene liquids (Schaufele, 1968). Thus, the 1122 cm^{-1} shoulder in the crystalline, or gel, form would reflect a small chain fragment uncoupled from the remainder of the all-trans form by one, or more, gauche rotations. The normal coordinate calculations (Snyder, 1967), however, support the contention that the 1122 cm^{-1} feature represents a gauche terminal methyl rotamer, where the feature is then characteristic of the entire chain, which includes the appropriately coupled trans and gauche segments. That is, calculations, in which gauche rotations are placed at various positions in the chain, indicate that only for a gauche rotation at the methyl end is the characteristic all-trans C–C stretching mode decreased in frequency (Snyder, 1967). Other single gauche bonds within the chain raise the frequency of the in-phase stretching mode relative to the all-trans mode. In summary at this point, gauche chain conformers are first formed in the gel state at temperatures less than the pretransition value; namely, −40, −40, and 5 °C for DMPC–H_2O, DPPC–H_2O, and DSPC–H_2O, respectively. The temperature-induced rotamers probably involve the terminal methyl and methylene fragments oriented toward the center of the bilayer.

The $\Delta H_{T<T_{m1}}$ values which are determined from the intensity changes of the Raman lines as a function of temperature serve as an additional probe for intramolecular disorder in the acyl chains of bilayer dispersions. For DMPC–H_2O and DPPC–H_2O gels, the Raman data yield ΔH enthalpy differences of 2.9 ± 0.6 and 3.4 ± 0.5 kcal/mol, respectively, or ~1.7 kcal/mol per acyl chain (based upon the DPPC value), between the all-trans and rotationally disordered isomeric states. (In this case we assume that a gauche conformation arising in one chain of a lipid molecule would create a suitable vacancy enabling the second chain to form an analogous gauche rotamer (Jackson, 1976).) This value may be compared with a calculation based upon spin label data in which an energy difference of 1.5 kcal/mol between the trans and gauche conformations about a carbon–carbon bond of liquid crystalline sodium decanoate–*n*-decyl alcohol–water bilayers was deduced (Seelig, 1971). Since Raman intensities for gas-phase *n*-butane result in an energy difference of 0.97 ± 0.1 kcal/mol between the trans and higher energy gauche conformer (Verma et al., 1974), the generally higher values found in the present gel systems and decanoate–liquid crystalline system probably reflect the effects from both intra- and intermolecular forces acting upon the potential function which governs internal rotation about a single carbon–carbon bond. If we accept a value of ~1.7 kcal/mol for an effective enthalpy difference between the trans and gauche conformers about a carbon–carbon single bond in the DMPC–H_2O and DPPC–H_2O gels, then the $\Delta H_{T<T_{m1}}$ data, summarized in Table II, suggest that the number of gauche bonds per molecule, n_g, of DMPC–H_2O, DPPC–H_2O, and DSPC–H_2O gels is approximately 2, 2, and 6, respectively. In assessing the conjectural nature of these values for n_g, we note that the tighter packing constraints imposed upon the gel assembly, as compared with the liquid crystalline–decanoate bilayers, may require values in excess of ~1.7 kcal/mol for the energy difference between acyl conformers. Although it is difficult to estimate for the gel state the extent of the domains in which the chains are disordered by rotational isomerization, the spectral data clearly imply for $T < T_{m1}$ a greater degree of disorder for DSPC–H_2O gels in contrast to DMPC–H_2O and DPPC–H_2O gels.

Since the gel to liquid crystalline phase transition involves an abrupt increase in the number of rotational isomers within the phospholipid acyl chains, it is of interest to estimate the number of gauche conformers contributing to the intramolecular disorder of the liquid crystalline state. Thus, for DPPC–H_2O, an enthalpy ΔH_{m2} of 9.7 ± 0.2 kcal/mol (Hinz and Sturtevant, 1972) leads to the formation of approximately

TABLE III: Phospholipid Transition Temperatures and Enthalpy Changes Determined by Hinz and Sturtevant (1972).

Phospholipid	Lower Transition		Main Transition	
	T_{m1} (°C)	ΔH_{m1} (kcal/mol)	T_{m2} (°C)	ΔH_{m2} (kcal/mol)
DMPC	13.5 ± 0.2	1.1 ± 0.2	23.70 ± 0.09	6.26 ± 0.18
DPPC	34.0 ± 0.2	2.3 ± 0.2	41.75 ± 0.06	9.69 ± 0.21
DSPC	49.1 ± 0.2	1.4 ± 0.2	58.24 ± 0.03	10.84 ± 0.17

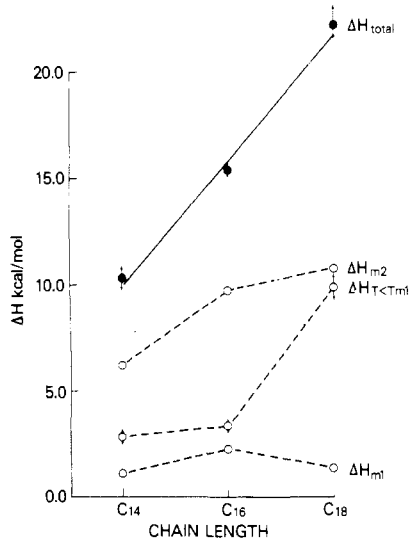

FIGURE 4: Enthalpy change (ΔH) dependence upon hydrocarbon chain length. Data for ΔH_{m1} and ΔH_{m2}, pretransition and main transition values, respectively, are taken from Hinz and Sturtevant (1972).

6.5 (9.7 kcal/mol/1.5 kcal/mol) new gauche bonds per molecule, n_g, where the liquid crystalline trans-gauche energy difference of 1.5 kcal/mol (Seelig, 1971) is used. To this value we add the number of gauche bonds formed at $T < T_{m1}$ and $T_{m1} < T < T_{m2}$. For $T_{m1} < T < T_{m2}$, the number of new gauche bonds formed is small since the enthalpies of these transitions probably arise from polar head group rearrangements accompanied to some extent by chain rotations (Hinz and Sturtevant, 1972; Yellin and Bulkin, 1976). Since two gauche bonds per molecule are estimated to be formed for $T < T_{m2}$, the total n_g for the liquid crystalline form of DPPC is then approximately 2 + 6.5, or 8–9 gauche bonds per molecule. This simple approach probably represents an upper limit as no corrections were applied here for van der Waals interactions occurring between acyl chains or polar head regions (Nagle, 1973). Analogous upper limits for n_g for liquid crystalline DMPC–H_2O and DSPC–H_2O are approximately 7 and 13–14 gauche bonds per molecule, respectively. These various estimates are also summarized in Table II.

Since the intramolecular disorder increases steeply between the C_{14}–C_{16} and C_{18} chain lengths for both the gel and liquid crystalline systems, the number of molecules forming the cooperative units associated with the primary phase transition would be expected to decrease significantly for the C_{18} species. Unfortunately, data relating to estimates of the cooperativity of the gel to liquid crystalline transition are difficult to quantitate. That is, the size of a cooperative unit is determined simply by the ratio of the van't Hoff enthalpy to the calorimetric enthalpy, where the van't Hoff heat is directly related to the width of the phase transition. A difficulty then arises in that the transition widths often appear to be critical functions of sample history. For example, the use of fluorescence probes to map the phase transition leads to estimates for the sizes of the cooperative units of about 10 to 25 molecules for the DMPC-, DPPC-, and DSPC–H_2O systems, respectively (Traüble, 1971). In contrast, the calorimetric widths, although varying between samples, are much narrower and lead to estimates of about 200, 70, and 80 molecules per cooperative unit for the C_{14}, C_{16}, and C_{18} phospholipid–water systems, respectively (Hinz and Sturtevant, 1972). It is of interest to note that ^{13}C NMR and ESR data give values of about 70 and 100 molecules for the cooperative units in the DMPC–H_2O system (Lee, 1975).

As shown in Figure 4, the various sets of data for $\Delta H_{T<T_{m1}}$, determined from Raman intensities, and for ΔH_{m1} and ΔH_{m2}, determined from calorimetric studies (Hinz and Sturtevant, 1972), display nonlinear relations with respect to phospholipid chain length. In contrast, a sum of the respective enthalpies, $\Delta H_{T<T_{m1}} + \Delta H_{m1} + \Delta H_{m2} = \Delta H_s$, suggests a nearly linear behavior, within the range of uncertainties of the measurements, in a correlation with chain length (Figure 4). An extrapolation of the plot for ΔH_s indicates that the sum of the enthalpies associated with the degree of inter- and intramolecular disorder relevant to the gel and liquid crystalline states is zero for phospholipid bilayers with C_{10}–C_{11} chain lengths. This value for chain length is consistent with the observation that at ordinary temperatures phospholipids with about ten carbon atoms or less per chain are not expected to form stable bilayers (see the discussion of Phillips, 1972).

In summary, the temperature dependence of the vibrational Raman spectra of phospholipid multilayers leads to a determination of the enthalpy differences between rotational isomers involving acyl chain conformations. These enthalpy differences appear useful for probing intramolecular disorder effects in the gel state. Extensions of these ideas to liquid crystalline forms and to multicomponent systems are currently under investigation and will be reported at a future date.

Acknowledgment

One of us (N. Y.) acknowledges the support provided by a National Institutes of Health Visiting Fellow appointment.

References

Bernstein, H. J. (1949), *J. Chem. Phys. 17,* 258.
Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.
Jackson, M. B. (1976), *Biochemistry 15,* 2555.
Jacobs, R. E., Hudson, B., and Andersen, H. C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3993.
Lee, A. G. (1975), *Prog. Biophys. Mol. Biol. 29,* 3.
Levin, I. W. (1969), *Spectrochim. Acta, Part A, 25,* 1157.
Levine, Y. K. (1972), *Prog. Biophys. Mol. Biol. 24,* 1.
Lippert, J. L., and Peticolas, W. L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1572.

Lippert, J. L., and Peticolas, W. L. (1972), *Biochim. Biophys. Acta 282,* 8.
McCammon, J. A., and Deutch, J. (1975), *J. Am. Chem. Soc. 97,* 6675.
Nagle, J. F. (1973), *J. Chem. Phys. 58,* 252.
Phillips, M. C. (1972), Progress in Surface and Membrane Science, Vol. 5, Danielli, J. F., Rosenberg, M. D., and Cadenhead, D. A., Ed., New York, N.Y., Academic Press p 139.
Rothman, J. E. (1973), *J. Theor. Biol. 38,* 1.
Schachtschneider, J. H., and Snyder, R. G. (1963), *Spectrochim. Acta 19,* 117.
Schaufele, R. F. (1968), *J. Chem. Phys. 49,* 4168.
Schindler, H., and Seelig, J. (1975), *Biochemistry 14,* 2283.
Seelig, J. (1971), *J. Am. Chem. Soc. 93,* 5017.
Snyder, R. G. (1967), *J. Chem. Phys. 47,* 1316.
Spiker, R. C., Jr., and Levin, I. W. (1975), *Biochim. Biophys. Acta 388,* 361.
Traüble, H. (1971), *Naturwissenschaften 58,* 277.
Traüble, H. (1972), *Biomembranes 3,* 197.
Verma, A. L., Murphy, W. F., and Bernstein, H. J. (1974), *J. Chem. Phys. 60,* 1540.
Yellin, N., and Bulkin, B. (1976), *Biochim. Biophys. Acta,* to be published.
Yellin, N., and Levin, I. W. (1976), to be published.

Nitrotyrosine Chelation of Nuclear Magnetic Resonance Shift Probes in Proteins: Application to Bovine Pancreatic Trypsin Inhibitor†

Timothy D. Marinetti, Grayson H. Snyder,‡ and Brian D. Sykes*

ABSTRACT: The interactions of Pr(III) and Eu(III) with specifically nitrated derivatives of the basic bovine pancreatic trypsin inhibitor have been studied using optical spectroscopy and nuclear magnetic resonance (NMR) at 250 and 270 MHz. Stability constants for proton and metal binding to nitrotyrosines 10 and 21 determined optically are in good agreement with those from NMR. Observation of the Eu(III)-induced NMR shifts of the ring protons of nitrotyrosine 21 allowed calibration of the magnetic interactions for this binding site. The Pr(III)-induced shifts for several resolved nonexchangeable backbone proton resonances were compared with calculated shifts using the known x-ray structure. With several simplifying assumptions, the Pr(III)-induced shifts were used to assign one α-CH and five NH protons to compatible sets of backbone positions which are consistent with the known pH dependence and resistance to exchange with solvent D_2O. Some of the more general aspects of lanthanide-induced shifts are discussed with reference to their use in proteins. Due to the complexities of the analysis of the shift data, the most straightforward use of this technique is in conjunction with the relaxation probe Gd(III) for measurement of intramolecular distances.

Nitrotyrosine has been proposed as a binding site for lanthanide ions as nuclear magnetic resonance structural probes of proteins in solution (Marinetti et al., 1975). Specific tyrosine nitration can be used to introduce a binding site into a protein at a known locus, extending the use of the lanthanide NMR[1] probes to proteins which do not possess a naturally occurring binding site for the lanthanide ions. This approach has been successfully applied to a study of the basic pancreatic trypsin inhibitor using Gd(III)-induced NMR relaxation (Marinetti et al., 1976). The major interest in the lanthanide ions is that the through-space dipolar interaction seems to account for most of the observed shifts and relaxation. Because this has a known and reasonably simple dependence on the relative position of the nucleus and the metal (Horrocks et al., 1973; Bleaney, 1972), the lanthanide-induced effects contain information on the three-dimensional structure of the molecule in solution. Lanthanide studies with lysozyme have helped assign some protein resonances to specific side chains (Campbell et al., 1973, 1975). These and other biological applications of the lanthanides have been reviewed (Reuben, 1975).

This manuscript extends the previous studies of lanthanide interactions with nitrotyrosine 21 of dinitro-BPTI. Stability constants for metal binding have been determined optically as an independent check on the values derived from NMR. The shifts induced by Pr(III) and Eu(III), attributed to nitrotyrosine 21 bound metal, have been analyzed quantitatively using the nitrotyrosine 21 ring protons to calibrate the magnetic interactions. Although the induced shifts of the ring protons behaved qualitatively like the model system, *N*-acetyl-L-3-nitrotyrosine ethyl ester, there was a difference in magnitude in the induced shifts between the protein and the model system. The shift results for the nonexchangeable NH resonances of the protein can be used, after making several assumptions, to tentatively assign five of the NH resonances to compatible sets of backbone positions. The assignments are consistent with other data such as the known pH dependence and resistance to exchange with solvent D_2O of the NH protons. Finally, the requirements for and limitations on the application of the

† From the Department of Biochemistry and MRC Group on Protein Structure and Function, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada. *Received July 26, 1976.* This paper taken in part from the Ph.D. thesis of T.D.M., Harvard University, 1975.

‡ Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305.

[1] Abbreviations used: NMR, nuclear magnetic resonance; UV, ultraviolet; ppm, parts per million; BPTI, bovine pancreatic trypsin inhibitor; ANTE, *N*-acetyl-L-3-nitrotyrosine ethyl ester; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid.