Calorimetric and Fluorescence Depolarization Studies on the Lipid Phase Transition of Bacteriorhodopsin–Dimyristoylphosphatidylcholine Vesicles†

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ABSTRACT: The thermotropic lipid phase transition of dimyristoylphosphatidylcholine vesicles reconstituted with bacteriorhodopsin was investigated as a function of the lipid to protein ratio by means of differential scanning calorimetry and fluorescence depolarization of the embedded probe 1,6-diphenyl-1,3,5-hexatriene. Two attractive features of this system are that the lipid phase transition induces lipid–protein segregation and that the state of aggregation of the protein is known. Above the lipid phase transition and above molar lipid to protein ratios of about 100, bacteriorhodopsin is monomeric. Well below the phase transition, bacteriorhodopsin is aggregated in a hexagonal protein lattice. With increasing amounts of incorporated bacteriorhodopsin, the calorimetric transition broadens, and a second component develops at a temperature which is lower than that of the unperturbed transition. The latter transition was assigned to the disaggregation of the bacteriorhodpsin lattice which occurs 6–7 °C below the phase transition of the protein-free lipids according to previous measurements. The van't Hoff enthalpy of the phase transition, as determined from the fluorescence depolarization of diphenylhexatriene, is in surprisingly good agreement with that obtained from differential scanning calorimetry over a wide range of lipid to protein ratios. The differential scanning calorimetry data can be simulated on the basis of a model which takes the protein segregation and crystallization specifically into account. The essential feature of this model is that calorimetrically detectable lipid melting occurs in the temperature region of the protein crystallization, since for the disaggregation of the close packed bacteriorhodopsin lattice additional lipids of an intermediate chain conformation are required.

The effect of intrinsic membrane proteins on the lipid phase transition has been studied calorimetrically as a function of the lipid to protein ratio in many reconstituted systems (Boggs et al., 1980; Curatolo et al., 1977; Gomez-Fernandez et al., 1980; Papahadjopoulos et al., 1975; Petri et al., 1980; Van Zoelen et al., 1978). Incorporation of only a very small amount of protein usually abolishes the pretransition. At lower lipid to protein ratios, the transition broadens and occasionally shifts to a different temperature. In most systems studied so far, the calorimetric enthalpy change and the cooperativity of the transition decrease with decreasing lipid to protein ratio.

One of the main difficulties in differential scanning calorimetry (DSC)[1] studies of this kind is that the state of aggregation of the proteins below and above the phase transition is usually not known, thus preventing a proper interpretation of the data. A different interpretation would be required, depending on whether the proteins are segregated in patches or are randomly dispersed as monomers below T_c. In this paper, we present the results of DSC and fluorescence depolarization measurements of the effect of bacteriorhodopsin on the phase transition of dimyristoylphosphatidylcholine (DMPC). Monomeric bacteriorhodopsin was incorporated in large (0.3-μm radius) unilamellar vesicles of DMPC at various lipid to protein ratios (Cherry et al., 1978; Heyn & Dencher, 1981; Heyn et al., 1981). These vesicles form an attractive model system for the study of lipid–protein interactions (Heyn et al., 1981). The physical properties of this vesicle system were previously studied in detail with freeze–fracture electron microscopy, X-ray diffraction, transient linear dichroism, circular dichroism, and fluorescence depolarization (Cherry et al., 1978; Heyn et al., 1981). The lipid phase transition induces a reversible aggregation of bacteriorhodopsin from a monomeric state well above T_c to a hexagonally aggregated state below T_c (Cherry et al., 1978; Heyn et al., 1981). We recently showed that bacteriorhodopsin is active as a light-driven proton pump both above and below T_c, suggesting that the hexagonal state of aggregation of the proteins is not required for function (Dencher & Heyn, 1979). Interestingly enough, the transition temperature for the formation of the protein lattice does not coincide with T_c of the lipids but lies 6–7 °C below T_c (Dencher & Heyn, 1979; Heyn et al., 1981). In agreement with these previous observations which were based on measurements of the rotational diffusion of BR and of the exciton CD spectra of BR, a broad second transition can be observed in the DSC curves presented in this paper, which occurs a few degrees centigrade below the normal transition temperature of DMPC and which increases in amplitude with increasing amounts of incorporated BR. The DSC data are interpreted in terms of a model in which a fraction of the lipids melts with reduced enthalpy change at the midpoint of the protein crystallization. These partially disordered lipids "solvate" the bacteriorhodopsin monomers which form during the disaggregation of the lattice. The calorimetric contribution from these lipids increases linearly with the mole fraction of bacteriorhodopsin. The remaining lipids melt with the normal enthalpy change at approximately the transition temperature of the protein-free lipids. This model may well be of value in other systems where lipid–protein segregation occurs.

The lipid phase transition was also monitored by means of the fluorescence depolarization of the probe DPH. The van't Hoff enthalpies obtained from a simple two-state analysis of

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[1] Abbreviations used: DSC, differential scanning calorimetry; CD, circular dichroism; BR, bacteriorhodopsin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphoshatidylcholine; L/BR, molar phospholipid to bacteriorhodopsin ratio; T_c, midpoint temperature of the lipid gel to liquid-crystalline phase transition.

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the fluorescence depolarization transition curves are in surprisingly good agreement with those obtained from DSC over the whole range of lipid to protein ratios studied. The results indicate that the fluorescence probe appears to sense correctly the change in the thermodynamic parameters of the lipid in its environment. The van't Hoff enthalpy, the calorimetric enthalpy, and the cooperativity of the transition all decrease when the amount of incorporated bacteriorhodopsin increases.

Materials and Methods

Chemicals. Dimyristoylphosphatidylcholine (DMPC) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Fluka. DMPC showed a single spot in thin-layer chromatography and was used without further purification.

Preparation of DMPC–Bacteriorhodopsin Vesicles. The preparation and properties of the DMPC–BR vesicles have been described in detail elsewhere (Cherry et al., 1978; Heyn et al., 1981; Heyn & Dencher, 1981). Protein-free vesicles were prepared in exactly the same way. All experiments were performed in 0.1 M sodium acetate buffer, pH 5.0. Protein concentrations were determined by the method of Hartree (1972). The results were corrected for a systematic error in the case of BR (Rehorek & Heyn, 1979). Phospholipid concentrations were determined by phosphorus analysis according to Ames & Dubin (1960) or by following a modified procedure of Hague & Bright (1941).

Vesicle Radius. Diffusion coefficients and Stokes radii were determined by quasi-elastic light scattering by using a laser as the light source and analyzing the scattered light spectrum produced by the Brownian motion of the vesicles. The light source was a Coherent Radiation argon ion laser operated at 488 nm. The correlation was carried out with a 24-channel Malvern digital autocorrelator. The correlator output was recorded on paper tape and processed on a Hewlett-Packard 9810 A computer. The correlation function could be fitted quite well by the sum of a single exponential and a constant. The average translational diffusion coefficient, D, of the vesicles was obtained from the exponent. For spherical vesicles, the Stokes radius, R, is related to D by

$$D = kT/(6\pi\eta R) \quad (1)$$

where η is the viscosity of the solvent, T the absolute temperature, and k Boltzmann's constant.

Steady-State Fluorescence Depolarization Experiments. DPH stock solutions were prepared in tetrahydrofuran. The label was incorporated in the vesicles by incubation for 30 min far above the T_c (Shinitzky & Barenholz, 1978). DPH:phospholipid ratios were usually 1:900 (mol/mol). Fluorescence depolarization measurements were carried out with a Schoeffel RRS 1000 recording fluorometer. The excitation beam was polarized by a Glan-Thompson polarizer. The emitted light was analyzed with Polacoat sheet polarizers. Excitation was at 360 nm, and emission was measured at 428 nm. The steady-state fluorescence anisotropy, $\bar{r}$, is defined by

$$\bar{r} = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (2)$$

where $I_\parallel$ and $I_\perp$ are the two fluorescence intensities with the analyzer parallel and perpendicular, respectively, to the vertical polarizer. Standard corrections for depolarization in the detection system were made (Azumi & McGlynn, 1962). The effect of depolarization due to light scattering was tested (Lentz et al., 1979). Dilution over a 50-fold range had no effect on the measured anisotropy values. The turbidity of the vesicle suspension was rather small. The lipid concentrations were typically on the order of 0.25 mg/mL. DPH in glycerol at −15 °C was used to test the performance of our apparatus. Anisotropy values of 0.39 were obtained. Increasing the label:phospholipid ratio to 1:100 had no significant effect on the $\bar{r}$ vs. temperature curve. The vesicle suspensions were contained in 1 × 0.4 cm stoppered fluorescence cuvettes. The temperature was measured by a thermistor immersed in the suspension above the light beam. Below 10 °C, the sample compartment was flushed with nitrogen to prevent condensation. The temperature was increased automatically at a rate of 12 or 18 °C/h. The two fluorescence intensities, $I_\parallel$ and $I_\perp$, were alternately recorded by rotating the analyzer over 90°. The two fluorescence intensities and the temperature were recorded as a function of time on a dual channel recorder. These data were then converted into $\bar{r}$ vs. T curves. Some of the data were also collected in a T geometry with two photomultipliers measuring $I_\parallel$ and $I_\perp$ simultaneously. The two measuring channels were balanced, with the polarizer in the horizontal position. The two fluorescence intensities were, in this case, converted electronically to anisotropy values, and the transition curves of $\bar{r}$ vs. temperature were directly available on an X–Y recorder. Some irreversible loss of fluorescence intensity occurred during a heating run, as is usually the case when DPH is irradiated (Shinitzky & Barenholz, 1978). The anisotropy depends, however, only on the ratio of the two fluorescence intensities. The change in $\bar{r}$ with temperature was completely reversible.

Calorimetry. All calorimetric scans were performed with a Privalov calorimeter (Privalov et al., 1975) using a scan rate of 2 K min^{-1}. The lipid concentration was in the range of 0.5–1 mg/mL of buffer. At least two runs were made with each sample. The reproducibility of the curves was excellent. The transition enthalpies were evaluated by using the following procedure. The calorimetric scans were digitized with a Hewlett-Packard plotter (Model 9872 A) connected to a Hewlett Packard 9845 A computer. The scans were replotted after computer subtraction of the instrumental base line, which had been determined before by scanning buffer vs. buffer. The base line was then linearly interpolated in the temperature range of the transition. The area of the calorimetric peak was determined by numerical integration. Molar calorimetric transition enthalpies (ΔH_{cal}) were calculated from the sensitivity of the instrument, which was obtained from an electrical calibration experiment, and from the phospholipid content. The degree of transition at temperature T, $\theta(T)$, was determined by integrating the excess heat capacity curves to T and dividing by the area under the curve:

$$\theta(T) = \left[\int^T \Delta C_P(T)\, dT\right] \Big/ \left[\int^\infty \Delta C_P(T)\, dT\right] \quad (3)$$

The midpoint temperature, T_c, of the transition is defined as the temperature at which $\theta = {}^1/_2$. The van't Hoff enthalpy, ΔH_{vH}, was obtained from the derivative of the $\theta(T)$ curve at the midpoint of the transition ($\theta = {}^1/_2$) in the following way:

$$\Delta H_{vH} = 4RT_c^2\left(\frac{d\theta}{dT}\right)_{T_c} \quad (4)$$

Clearly ΔH_{vH} is a measure of the steepness of the transition. The ratio of the van't Hoff and the calorimetric enthalpies

$$n = \frac{\Delta H_{vH}}{\Delta H_{cal}} \quad (5)$$

provides an estimate for the size n of the cooperative unit for the transition (Mabrey & Sturtevant, 1978).

Table I: Dependence of Vesicle Radius, Transition Temperature, Enthalpy Change, and Size of the Cooperative Unit on the Lipid to Bacteriorhodopsin Ratio

L/BR ratio	vesicle radius, R (nm)	$\Delta H_{fl,vH}$ (kcal/mol)	$T_{c,fl}$ (°C)	ΔH_{cal} (kcal/mol)	$\Delta H_{cal,vH}$ (kcal/mol)	size of cooperative unit, n
91	62	66.5	22.8	4.4	54.5	12.4
188	290	80.0	23.0	6.3	68	10.8
316	279	113.1	23.2	6.0	113	18.8
552	220	182.6	23.3	7.3	134	18.4
∞	280	305.9	24.1	7.6	287	37.8

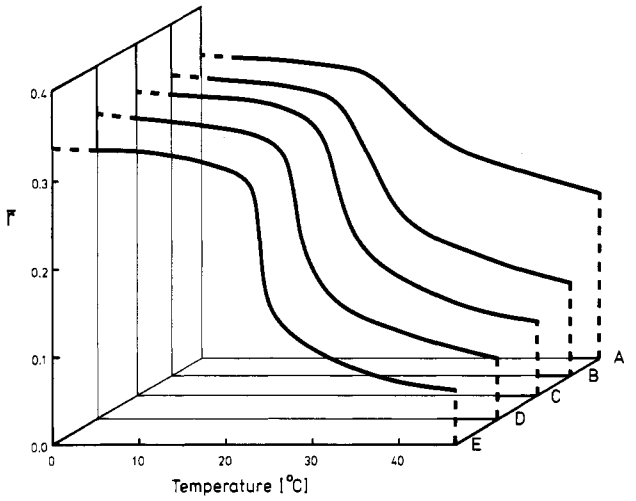

FIGURE 1: Temperature dependence of the steady-state fluorescence anisotropy $\bar{r}$ of DPH embedded in bacteriorhodopsin–DMPC vesicles of molar phospholipid to protein ratios of 91 (A), 188 (B), 316 (C), 552 (D), and ∞ (E).

Results and Interpretation

Vesicle Radius. From our previous electron microscopy work, we know that these vesicles are predominantly unilamellar and have radii between 0.1 and 0.3 μm (Cherry et al., 1978). For the present set of experiments, it was important to know if the vesicle radius varied with the lipid to protein ratio. The mean vesicle radius R was determined by quasi-elastic light scattering as explained under Materials and Methods. The results are displayed in the second column of Table I. The mean radius is quite large (~0.25 μm) and independent of the lipid to protein ratio except for the sample with the highest protein content.

Fluorescence Depolarization. Figure 1 shows the results of fluorescence depolarization experiments with BR–DMPC vesicles which were incubated with the probe DPH. The measurements were performed with the same vesicles as were used in the DSC experiments. The lipid phase transition, which is quite steep in the absence of bacteriorhodopsin, gradually broadens as more and more protein is incorporated. Far below T_c, a plateau of about 0.35 is reached in the fluorescence anisotropy which is practically independent of the lipid to protein ratio. This is reasonable, since no matter what value this ratio has bacteriorhodopsin is crystallized in a lattice at these temperatures and the DPH trapped in the lattice has about the same high $\bar{r}$ value as the DPH in the lipid gel phase (Heyn et al., 1981). Above T_c, the anisotropy values increase markedly with decreasing lipid to protein ratio. This phenomenon can be explained in terms of a protein-induced increase in the bilayer viscosity and order parameter or in a decrease in the fluorescence lifetime (Heyn, 1979; Heyn et al., 1981). The transition curves of Figure 1 were analyzed in the following way. As is done in the determination of the van't Hoff enthalpy from DSC data, it is assumed that the lipids participate in a simple two-state equilibrium between

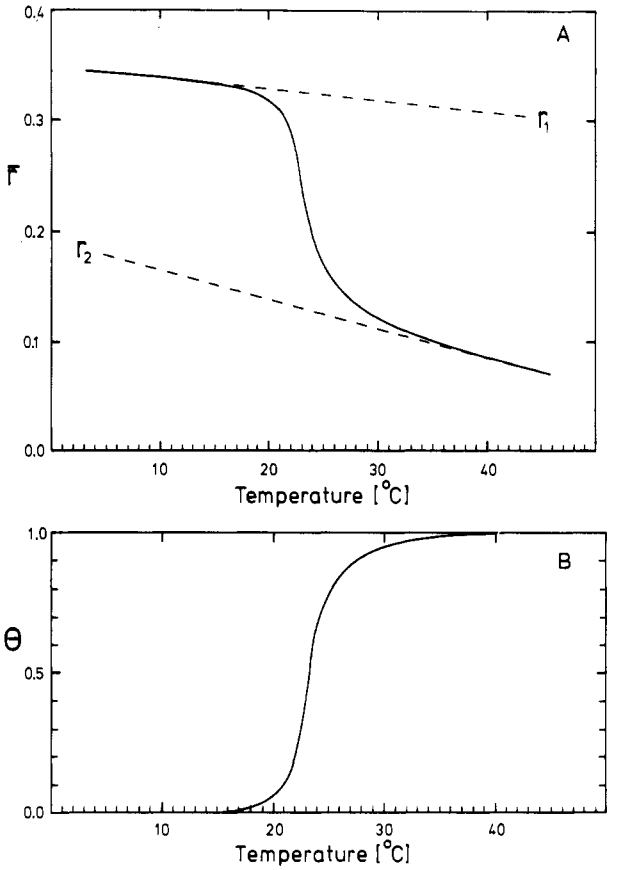

FIGURE 2: (A) Temperature dependence of the DPH fluorescence anisotropy $\bar{r}$ in BR–DMPC vesicles of molar lipid to protein ratio 552. r_1 and r_2 are the low- and high-temperature tangents, respectively, to the transition curve. (B) Normalized transition curve obtained from the data in (A) by defining $\theta = (r_1 - \bar{r})/(r_1 - r_2)$.

a gel phase in which DPH has a high anisotropy and a liquid-crystalline state with a low anisotropy. It is furthermore assumed that the anisotropy curve $\bar{r}(T)$ accurately reflects this transition. The degree of transition $\theta(T)$, defined as the fraction of lipids in the liquid-crystalline state, can then be obtained in the following way (see Figure 2). The low- and high-temperature $\bar{r}(T)$ data are linearly extrapolated forward and backward in temperature as shown in Figure 2A. At each temperature, $\theta(T)$ is then given by the ratio $(r_1 - \bar{r})/(r_1 - r_2)$. The θ curve corresponding to the data of Figure 2A is shown in Figure 2B. Since the van't Hoff enthalpy is determined by the slope of the $\theta(T)$ curve at the midpoint of the transition (see eq 4), the linear extrapolations of the $\bar{r}(T)$ data have to be performed over only a small range of temperatures. Even if a linear extrapolation of $\bar{r}(T)$ is not justified over an extended temperature range, such a local linearization in the neighborhood of T_c is acceptable. T_c was determined as the temperature at which $\theta = {}^1/_2$. The van't Hoff enthalpy was determined from the slope of the transition curve at T_c. The results obtained in this way by fluorescence measurements ($\Delta H_{fl,vH}$, $T_{c,fl}$) are presented in Table I. Whereas $T_{c,fl}$ decreases

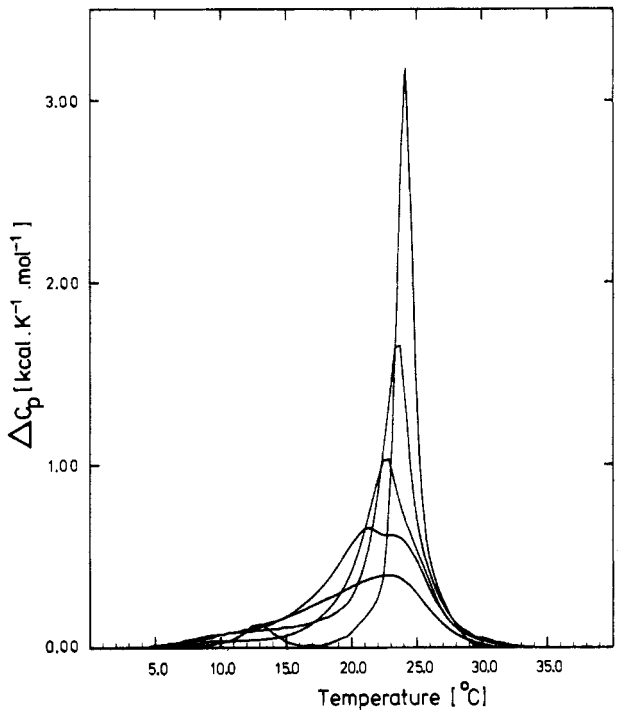

FIGURE 3: Excess heat capacity curves for BR–DMPC vesicles of varying molar lipid to protein ratios. With increasing broadening of the transition, the molar lipid to protein ratios are ∞, 552, 316, 188, and 91. The pretransition is only observed for the protein-free vesicles.

only slightly, $\Delta H_{fl,vH}$ decreases markedly with decreasing L/BR. The small effect of bacteriorhodopsin on T_c has been noted before (Heyn et al., 1981). In accordance with the pronounced broadening of the transition curves of Figure 1, the van't Hoff enthalpy, which is a measure of the width of the transition (eq 4), decreases considerably as the amount of incorporated bacteriorhodopsin increases. The slight break in the transition curve for the pure lipids around 10 °C (Figure 1, E) may be due to the pretransition.

Differential Scanning Calorimetry Data. The DSC results for a series of vesicles with molar lipid to protein ratios varying between 91 and infinity are shown in Figure 3. Already at a molar lipid to protein ratio of 552, the pretransition is abolished. With increasing amounts of incorporated bacteriorhodopsin, the transition broadens, and a shoulder or second component develops at the low-temperature side.

From these data, the calorimetric enthalpy change (ΔH_{cal}), the van't Hoff enthalpy change ($\Delta H_{cal,vH}$), and the cooperativity of the transition were calculated as explained under Materials and Methods. The results are presented in Table I. Both the enthalpy change and the cooperativity decrease with decreasing lipid to protein ratio. For the protein-free vesicles, the calorimetric enthalpy change has about the value expected for DMPC (Mabrey & Sturtevant, 1978).

Interpretation of the DSC Data. The DSC transition curves were simulated in terms of a model which takes into account the information which we obtained previously on the formation of the BR lattice (Dencher & Heyn, 1979; Heyn et al., 1981). From the temperature dependence of the CD spectra and of the rotational diffusion of BR, we found that the crystallization of BR in these vesicles occurs well below the T_c of the bulk lipids and has a midpoint temperature of 17.5 °C. From the slope of the CD transition curves for the formation of the protein lattice, one can estimate a van't Hoff enthalpy of about 55 kcal/mol. These normalized CD transition curves are almost symmetric with respect to the midpoint and approximately independent of the lipid to protein ratio (Heyn et al., 1981). The disaggregation of the bacteriorhodopsin lattice around 17.5 °C leads to the formation of small BR aggregates and monomers. In the lattice, the BR molecules are densely packed with many direct protein–protein contacts and only about ten lipid molecules per protein. From the surface areas of BR and DMPC, it can be estimated that about 30 DMPC molecules are required to form a single lipid layer around BR (see Discussion). In the course of the lattice disaggregation, therefore, the BR molecules have to acquire a large number of additional lipids around them, which are supplied by the pool of gel lipid. These lipids may be regarded as forming a kind of solvation layer around the proteins and will be called for convenience "solvation lipids". It is now assumed that these lipids which have to accommodate themselves to the rough irregular surface of the proteins melt only partially in this process, acquiring a conformation which is intermediate between the all-trans and the liquid-crystalline state. This fraction of the lipids, which increases linearly with the bacteriorhodopsin to lipid ratio, has accordingly a calorimetric enthalpy change which is smaller by a factor F ($0 < F < 1$) than that of the protein-free lipids. The remaining lipids (bulk lipids) are assumed to melt at approximately the usual transition temperature of DMPC with the normal calorimetric enthalpy change. The solvation lipids which partially melt below T_c are supposed to remain in this intermediate conformation above T_c; i.e., no additional melting takes place around T_c. Rapid exchange between bulk and solvation lipids is assumed to occur above T_c. We make the reasonable assumption that the disaggregation of the protein lattice and the melting of the solvation lipids are closely coupled, i.e., that the degree of transition of the two reactions is equal. This allows us to use the thermodynamic parameters of the protein disaggregation (55 kcal/mol; 17.5 °C) for the melting of the solvation lipids. The main parameters entering this model are F and the number of solvation lipids per BR, m. With these assumptions, the calorimetric enthalpy change is given by

$$\Delta H_{cal} = \frac{BR}{L} m \Delta H_{1,cal} + \left(1 - \frac{BR}{L} m\right) \Delta H_{2,cal} = \left[1 - \frac{BR}{L} m(1 - F)\right] \Delta H_{2,cal} \quad (6)$$

and the degree of transition $\theta(T)$ by

$$\theta(T) = \frac{1}{\Delta H_{cal}} \left[\frac{BR}{L} m \Delta H_{1,cal} \theta_1(T) + \left(1 - \frac{BR}{L} m\right) \Delta H_{2,cal} \theta_2(T)\right] \quad (7)$$

$\Delta H_{1,cal}$ is the calorimetric enthalpy change for the solvation lipids; $\Delta H_{2,cal}$ is that for the bulk lipids. $F = \Delta H_{1,cal}/\Delta H_{2,cal}$. θ_1 and θ_2 are the degrees of transition, respectively, for the solvation lipids melting at T_1 (17.5 °C) with $\Delta H_{1,vH}$ of 55 kcal/mol and for the bulk lipid transition occurring at T_2 with $\Delta H_{2,vH}$ (the latter two parameters depend on the lipid to protein ratio):

$$\theta_1(T) = \frac{1}{1 + \exp[-(\Delta H_{1,vH}/R)(1/T - 1/T_1)]} \quad (8)$$

and

$$\theta_2(T) = \frac{1}{1 + \exp[-(\Delta H_{2,vH}/R)(1/T - 1/T_2)]} \quad (9)$$

From eq 6, we note that ΔH_{cal} is expected to decrease with decreasing L/BR as is observed. In most of the previous work, it was assumed that the membrane proteins are surrounded

Table II: Parameters for the Fit of the Calorimetric Transition Curves of Figure 4 to Equation 7 of the Model

L/BR ratio	T_1 (°C)	$\Delta H_{1,vH}$ (kcal/mol)	T_2 (°C)	$\Delta H_{2,vH}$ (kcal/mol)	solvation lipids per BR, m	F ($=\Delta H_{1,cal}/\Delta H_{2,cal}$)
91	17.5	55	22.3	80	50	0.7
188	17.5	55	22.3	80	60	0.7
316	17.5	55	22.9	115	60	0.7
552	17.5	55	23.5	150	60	0.7

by boundary lipids which do not participate in the phase transition at all. The linear decrease in ΔH_{cal} with the mole fraction of protein is then simply due to the increase in the amount of boundary lipid. In our model, it is a consequence of the assumption that with higher protein content a larger fraction of the lipids is required to solvate the monomeric proteins which are formed when the lattice disassembles. Since the enthalpy change for this partial melting to a state of intermediate chain order is less than that for the bulk lipids ($F < 1$), ΔH_{cal} decreases with increasing mole fraction of protein. Equation 7 describes the superposition of the two transitions at T_1 and T_2, which are weighted with the respective calorimetric enthalpies $\Delta H_{1,cal}$ and $\Delta H_{2,cal}$. At first sight, it appears that eq 6 and 7 contain too many adjustable parameters. Most of them however, are either accurately known or lie within a narrow band width of possible values. As noted above, T_1 and $\Delta H_{1,vH}$ for the lattice formation have values of 17.5 °C and 55 kcal/mol. T_2 is approximately known from the fluorescence depolarization data and from the position of the higher temperature component in the DSC curve. T_2 was therefore varied within a very narrow range. The value of $\Delta H_{2,vH}$ is expected to be close to the van't Hoff enthalpy determined from the fluorescence experiments since DPH only senses the bulk lipid phase transition (see Discussion). For $\Delta H_{2,cal}$, we take the calorimetric enthalpy change for the vesicles without bacteriorhodopsin. This leaves us with the completely unknown parameters m, the number of lipids per BR which partially melt at T_1, and $F = \Delta H_{1,cal}/\Delta H_{2,cal}$, the factor by which the enthalpy change of the first transition is reduced with respect to that of the second. Figure 4 shows that the experimental $\theta(T)$ curves, obtained by integration from the experimental $\Delta C_P(T)$ curves of Figure 3, can be fitted reasonably well by using the model described above. The fit parameters are summarized in Table II. The values for T_2 are only slightly lower than those from fluorescence depolarization (Table I) and decrease somewhat with decreasing L/BR. The same trend is also clearly present in the transition temperature results from fluorescence depolarization (Table I) and confirms our earlier results (Heyn et al., 1981). The values for $\Delta H_{2,vH}$ show the expected increase with increasing L/BR and are not much different from the van't Hoff enthalpies determined from fluorescence depolarization (Table I). Whereas T_1, T_2, $\Delta H_{1,vH}$, $\Delta H_{2,vH}$, and $\Delta H_{2,cal}$ were more or less fixed by the results from other methods, no a priori values for the parameters m and F were known. From Table II, one notes that over the whole range of L/BR ratios the data could be fitted with a single value of 60 for m, except for the lowest L/BR ratio where $m = 50$ gave a slightly better fit. Since the improvement in fit is only marginal, one may conclude that about 60 lipid molecules are involved in the solvation of one BR molecule regardless of the mole fraction of BR. Likewise the data for the various L/BR ratios could be fitted with a single value of 0.7 for F. Since $\Delta H_{2,cal}$ is independent of the lipid to protein ratio, this means that the melting enthalpy change for the solvation lipids does not depend on this ratio either.

Discussion

The model presented provides a possible way of analyzing

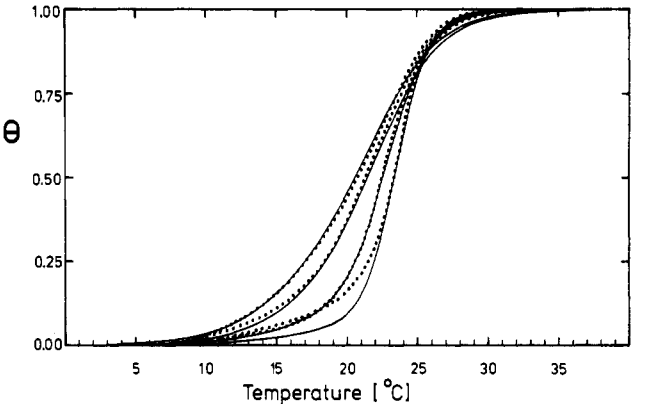

FIGURE 4: Fit of the experimental calorimetric transition curves for BR–DMPC vesicles (---) by the model described in the test (—). With increasing steepness of the transition (from left to right), the molar lipid to bacteriorhodopsin ratios are 91, 188, 316, and 552. The parameters used in the fit are presented in Table II.

DSC curves for systems where lipid–protein segregation and subsequent protein lattice formation occur. More complicated models, which will certainly improve the quality of the fit, are not justified at this time because of the limited accuracy of the data. For simplicity, the lipids which are trapped in the protein lattice were not considered in the simulation (about 10 per BR). Using the value of 60 for m, we can calculate the number of solvation layers around BR from the surface areas of BR (875 Å^2) and DMPC (60.8 Å^2 at 37 °C; Curatolo et al., 1977). We conclude that between one and two layers of DMPC are involved in the solvation of one BR (one layer, 30 lipids; two layers, 73 lipids). At a L/BR ratio of 91, one is close to the condition where some BR aggregation occurs even above the phase transition (R. J. Cherry and R. E. Godfrey, unpublished results). This may be the reason that at this ratio a value of 50 was obtained from m. The decrease of ΔH_{cal} with increasing amount of incorporated protein is often interpreted in terms of boundary lipids. These lipids in the immediate vicinity of membrane proteins are assumed not to participate in a calorimetrically detectable phase transition and are said to be "taken out of the transition" by the proteins. The number of boundary lipids is obtained from the slope of the plot of ΔH_{cal} vs. the mole fraction of protein. Such a simple approach is not warranted with the present system, since a second component develops in the ΔC_P curve at low lipid to protein ratios. The value for m of about 60 cannot therefore be directly compared to the number of boundary lipids obtained in this way for other systems. In our approach, the m lipids per BR molecule do participate in a melting transition at a lower temperature than the bulk lipids and with a reduced ΔH. They are related to the boundary lipids though in two respects: they are in a different conformation than the bulk lipids, and they do not contribute to the calorimetric transition at T_2. We may say that they were prevented from participating in the bulk lipid transition by their participation in the lower lying protein disaggregation. Since lipid–protein segregation is not uncommon (Kleemann & McConnell, 1976; Höchli & Hackenbrock, 1976; Letellier et al., 1977), similar

considerations may well be of value with other systems as well.

From Figure 2B we note that the transition curve obtained from fluorescence depolarization is rather symmetrical and provides no evidence for a transition around 17.5 °C. The same holds for the other transition curves of Figure 1. We may therefore conclude that DPH senses only the phase transition of the bulk lipids. Before this conclusion is made, however, the question must be answered why it is that DPH does not sense the transition at T_1, in spite of the fact that a partial melting to an intermediate conformation is supposed to occur at this temperature in which an appreciable fraction of the lipids participate. The explanation follows from the observation that the DPH in the lipid region adjacent to BR is strongly quenched due to energy transfer to the retinal chromophore of BR (M. Rehorek, M. P. Heyn, and N. A. Dencher, unpublished experiments). The range of the energy transfer is about 46 Å and therefore extends over a thickness of more than three lipid layers into the lipid phase. Since the contribution to $\bar{r}$ is proportional to the fluorescence lifetime, the DPH molecules in a lipid neighborhood of 46 Å around BR are masked, and only those in the bulk lipid phase contribute to the signal. This argument also justifies the use of a two-state model in the evaluation of the anisotropy data. We must therefore expect that $\Delta H_{fl,vH}$ equals $\Delta H_{2,vH}$. From a comparison of Tables I and II, we conclude that this is indeed the case to a good approximation. One argument against the use of labels in membrane biophysics is that they possibly change the properties of the bilayer which one wishes to measure. In the present case, the fluorescence anisotropy responds to changes in lipid order and viscosity in the label's own environment, which is possibly perturbed with respect to label-free regions. It is therefore reassuring that over a broad range of lipid to protein ratios the DPH anisotropy leads to the same thermodynamic parameters for the bulk lipid phase transition as those obtained from calorimetry (T_2, $\Delta H_{2,vH}$). The overall transition is of course broader than the transition at T_2. As a result, $\Delta H_{cal,vH}$, which is a measure of the width of the complete transition, is expected to be smaller than $\Delta H_{2,vH}$. From a comparison of Tables I and II, we note that this is indeed the case. One might have expected that $\Delta H_{cal,vH}$ would decrease more with decreasing lipid to protein ratio than $\Delta H_{2,vH}$. This does not appear to be the case and may be due to the limited accuracy of the data.

Evidence for the existence of an intermediate chain conformation for the solvation lipids was obtained from fluorescence depolarization studies with vesicles in which the acceptor retinal had been removed by bleaching (M. Rehorek, M. P. Heyn, and N. A. Dencher, unpublished experiments). In these vesicles, no energy transfer to retinal occurs, and the observed steady-state anisotropy is due to DPH molecules in both the bulk and solvation regions. In the presence of bleached BR above T_c, $\bar{r}$ is considerably higher than in protein-free vesicles, which is mainly due to a protein-induced increase in the lipid order parameter. Below T_c, the opposite effect was observed. Further support for the concept adopted here, that the solvation lipids are in an intermediate chain conformation, comes from Raman spectroscopy studies with DMPC–myelin proteolipid apoprotein vesicles (Curatolo et al., 1978). In comparison with DMPC alone, the DMPC acyl chains in this system possess on the average more gauche conformation below T_c and more trans conformation above T_c. Further evidence for this notion was obtained with Ca^{2+}-ATPase–DPPC vesicles. In this system, protein-rich patches, which are formed by phase separation, melt well below the bulk lipid phase transition (Gomez-Fernandez et al., 1980). From the temperature dependence of the 4.2-Å reflection in the X-ray diffraction pattern, it was concluded that the amount of all-trans gel lipid starts to decrease about 10 °C below T_c, in parallel with the melting of the protein patches (Hoffmann et al., 1980). Similar conclusions were reached in recent Raman studies on DPPC–glycophorin vesicles (Taraschi & Mendelsohn, 1980). In this work, the presence of a class of perturbed phospholipids was detected which undergo a broad melting transition with the formation of gauche rotamers about 15 °C below T_c.

It is conceivable that the protein crystallization contributes in a direct way to ΔC_P rather than in the indirect way suggested here. Calorimetric experiments with purple membrane suspensions show the presence of a small transition with a transition enthalpy of about 7 kcal/mol (referred to moles of bacteriorhodopsin monomers) at 80 °C (Jackson & Sturtevant, 1978). Recent X-ray and CD experiments suggest that the long-range hexagonal order is lost above this temperature but that the BR molecules are still arranged in trimers (Hamanaka et al., 1980). The observed calorimetric enthalpy change therefore refers only to a partial protein disaggregation but nevertheless gives an idea of the expected order of magnitude for the complete disaggregation. Conversion of the enthalpy value of 7 kcal/mol to a moles of lipid basis by using the lipid to protein ratios of this study shows that the contribution of the protein–protein interactions to ΔC_P is so small that they may be neglected in our model.

The broadening of the bulk lipid phase transition as expressed by the dependence of $\Delta H_{2,vH}$ on the lipid to protein ratio is most likely the result of several factors. With increasing mole fraction of BR, the number of lipids than can participate in the cooperative melting at T_2 is reduced. At high enough protein content, the average size of lipid regions which are free of protein and solvation lipids becomes smaller than the cooperative unit for the protein-free lipids, and the van't Hoff enthalpy will decrease. It is well-known that the enthalpy change of the lipid phase transition also decreases with decreasing vesicle radius (Grünewald et al., 1979). This effect starts to be important at vesicle radii which are much smaller than those reported here (Table I). In addition, the vesicle radius is practically independent of the L/BR ratio except at a ratio of 91.

The cooperativity of the lipid phase transition is rather low, even in the vesicles without BR. Impurities have a dramatic lowering effect on the van't Hoff enthalpy and on the cooperativity of the lipid phase (Albon & Sturtevant, 1978). It is possible that the small amount of residual detergent (one Triton molecule per two BR molecules), which was also present in the vesicles without BR, is responsible for the low cooperativity.

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Thermal Behavior of Stearoylsphingomyelin–Cholesterol Dispersions†

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ABSTRACT: The thermotropic behavior of aqueous dispersions of stearoylsphingomyelin–cholesterol mixtures was examined by high-sensitivity differential scanning calorimetry. When less than 20 mol % cholesterol was mixed with the sphingomyelin and the samples were held at room temperature for 7–9 days before the initiation of calorimetric measurements, a sharp endotherm at 56–57 °C and a broad endotherm at 35–50 °C were observed. In addition, samples containing 15–20 mol % stearol exhibited a sharp endotherm at 43–45 °C. If samples were held at room temperature for less than 2 h before the initiation of calorimetric analysis, the 56–57 °C endotherm was usually not seen. Instead, a combination of broad and sharp endotherms over the range of 35–50 °C was observed. Occasionally, exotherms were also observed within this temperature range. These results, along with those from previous studies, imply that a cholesterol-rich phase coexists with a cholesterol-poor phase in which the sphingomyelin molecules may exist in two distinctly different gel states.

Since sphingomyelin and cholesterol comprise a significant fraction of the lipids present in many mammalian cells (Rouser et al., 1968), the study of their mixtures is pertinent to the understanding of the structure of biological membranes. On the basis of chemical and physical evidence, it has been suggested that sphingomyelin and cholesterol exhibit a preferential interaction (Vandenheuvel, 1963; Patton, 1970; Demel et al., 1977). For these reasons a systematic investigation using differential scanning calorimetry of aqueous dispersions of mixtures of synthetic sphingomyelins with cholesterol was initiated. Results of experiments with sphingomyelins containing palmitoyl or lignoceryl fatty acyl residues have already been reported (Estep et al., 1979). Due to the unexpected behavior of dispersions containing stearoylsphingomyelin (Estep et al., 1980), studies of mixtures of this phospholipid with cholesterol are now being reported separately.

Experimental Procedures

Materials. DL-*erythro*-*N*-Stearoylsphingosinephosphorylcholine (stearoylsphingomyelin) was supplied by Professor D. Shapiro (Weizmann Institute of Science, Rehovot, Israel) and Dr. C. T. Schmidt (Department of Biochemistry, University of Virginia, Charlottesville, VA). The lipid was purified and analyzed as described previously (Estep et al., 1980). Only sphingomyelin which readily exhibited a single endotherm at 57 °C when dispersed into aqueous solution was utilized in

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