Intrinsic Proteins and Their Effect upon Lipid Hydrocarbon Chain Order†

David A. Pink,* Alexandros Georgallas, and Dennis Chapman

ABSTRACT: We present evidence that at temperatures greater than their main transition temperature, phospholipid molecules that are trapped within clusters of intrinsic molecules such as polypeptides or proteins have the ends of their hydrocarbon chains more statically disordered than those of lipid molecules far from such intrinsic molecules. We have constructed a model in which the lipids are divided into three populations: (i) those that are not adjacent to any protein ("free" lipids), (ii) those that are adjacent to only one protein ("adjacent" lipids), and (iii) those that are "trapped" between two or three proteins. We applied this model to study deuterium nuclear magnetic resonance of dimyristoyl-3-*sn*-phosphatidylcholine (DMPC) bilayers containing gramicidin A′ or cytochrome oxidase and found that while the methyl groups of adjacent lipids are slightly more statically ordered than those of free lipids, the methyl groups of trapped lipids are more statically disordered than those of free lipids. We propose a physical explanation for this and show that phosphorus-31 nuclear magnetic resonance data for DMPC–cytochrome oxidase bilayers can be understood as a consequence of changes in the polar region of only trapped lipids.

There is at present much interest in the interactions between intrinsic proteins and lipid within the lipid bilayer structures of natural and model biomembranes (Chapman et al., 1979). Recent deuterium nuclear magnetic resonance (^{2}H NMR) studies have emphasized the rapid exchange of the lipid adjacent to the protein with the "bulk" lipid, the lack of evidence of stoichiometric protein–lipid complexes, and the increase in lipid chain "static disorder" that occurs with increasing protein concentration (Kang et al., 1979; Rice et al., 1979a; Seelig & Seelig, 1980). Some proteins [e.g., cytochrome oxidase, sarcoplasmic reticulum adenosinetriphosphatase (ATPase), myelin proteolipid apoprotein (PLA), and lipophilin] when incorporated into fluid dimyristoyl-3-*sn*-phosphatidylcholine (DMPC) bilayers (at 30 °C) give deuterium quadrupole splitting values, $\Delta\nu_Q$, associated with the end methyl group of the hydrocarbon chains, that appear to decrease monotonically as the protein concentration increases (Kang et al., 1979; Rice et al., 1979a). This result, i.e., that within a lipid bilayer that is above its "main" transition temperature, T_c, the presence of an intrinsic protein causes an increase in chain disorder, at least near the terminal methyl group, has been contrasted with the effect produced by cholesterol molecules [see Rice et al. (1979a)]. The effect due to cholesterol appears to be the opposite to that which occurs with intrinsic proteins. The quadrupole splittings for various positions along the chain increase with increasing cholesterol concentration so that above the T_c cholesterol appears to induce more "static order" in the chains (Jacobs & Oldfield, 1979).

As an explanation for the contrasting results between cholesterol and intrinsic proteins it has been suggested (Kang et al., 1979; Rice et al., 1979a) that the surfaces of the proteins in contact with lipids are sufficiently "rough" so as to preferentially constrain a lipid chain adjacent to the protein to be in states with a number of gauche bonds. It is important to note, however, that such considerations of the effects of proteins or polypeptides upon lipid chain order usually ignore many-body effects. Thus, for example, this suggestion that a protein causes adjacent lipid chains to be disordered takes into account only direct two-body interactions. The possible consequences of many-body effects, which might come about when the protein/lipid ratio is high, are usually ignored. This has been mentioned elsewhere (Pink & Chapman, 1979).

In the present paper we develop this point further. We explicitly consider the possible effects and consequences upon hydrocarbon chain order parameters of the "trapping" of lipids between proteins.

We shall consider DMPC bilayers at 30 °C containing first the polypeptide gramicidin A′ and then the protein cytochrome oxidase. We shall show that we can understand the concentration dependence of $\Delta\nu_Q$ by relating it to the populations of lipid adjacent to, or trapped between, these intrinsic molecules, the population changing with polypeptide or protein concentration within the lipid bilayers. We shall then suggest a physical mechanism for the effects proposed. Finally we shall comment on effects associated with perturbations of the lipid polar region as a consequence of increasing intrinsic molecule (protein or polypeptide) concentration.

We emphasize here that the application of our analysis assumes that the DMPC–gramicidin A′ and the DMPC–cytochrome oxidase systems are bilayers for sufficiently high concentrations of the polypeptide and the protein. In practice this means that we assume that a bilayer structure exists for gramicidin A′ concentrations up to ~50 wt % and cytochrome oxidase concentrations of ~90 wt %.

Theory

Lattice Model. We are going to construct a model of intrinsic molecules such as proteins or polypeptides that are randomly distributed in the plane of a DMPC bilayer. Such a distribution should be appropriate for such a bilayer at 30 °C, which is above the lipid main transition temperature, T_c. We shall consider three populations of lipid molecules: those not adjacent to any intrinsic molecule, those adjacent to only one intrinsic molecule, and those "trapped" between two or more such molecules. We shall then propose that the interactions between the lipids and the intrinsic molecules are such that the end-chain methyl groups of the lipid molecules in the trapped population are significantly more disordered than those in the other populations. We shall fix the free parameters of

† From the Theoretical Physics Institute, St. Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 1C0 (D.A.P. and A.G.), and the Department of Biochemistry and Chemistry, Royal Free Hospital, School of Medicine, University of London, London WC1N 1BP, England (D.C.). *Received April 8, 1981; revised manuscript received July 9, 1981.* Supported by the Natural Sciences and Engineering Research Council of Canada and St. Francis Xavier University (D.A.P. and A.G.) and the Wellcome Trust, the Medical Research Council, and the Cancer Research Campaign (D.C.).

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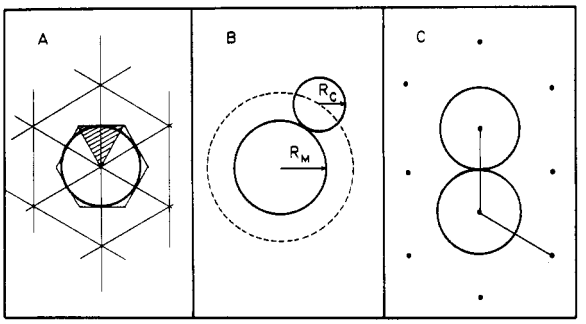

FIGURE 1: (A) Portion of a triangular lattice with one intrinsic molecule of circular cross section located at a site. The hexagon surrounding it is the unit cell. (B) An intrinsic molecule of radius R_M with one adjacent lipid chain of radius R_C. Adjacent chains arrange themselves so that their centers lie on the dashed circle of circumference $2\pi(R_M + R_C)$. (C) Two adjacent intrinsic molecules showing one dangling bond and one paired bond.

the theory by studying the case of gramicidin A′ in DMPC at 30 °C and then use these parameters to predict the effect of cytochrome oxidase.

In order to model a lipid bilayer, at temperatures above the T_c, into which are incorporated intrinsic molecules such as proteins or polypeptides, we shall assume that the intrinsic molecules are restricted to lie on the sites of a triangular lattice containing N sites and that the remainder of the space is filled with lipid molecules. The triangular lattice thus represents the two lateral dimensions of the bilayer. We assume that we have periodic boundary conditions so that all sites have six nearest-neighbor sites. We then distribute N_M intrinsic molecules at random on the N sites, since there is no indication of any phase separation in such a system for temperatures $T > T_c$. We define $C = N_M/N$ to be the concentration of intrinsic molecules with respect to the sites that they can occupy.

To compute how many lipid molecules are contained within the bilayer, we must develop a model that describes how the lipids pack in the space between the random distribution of intrinsic molecules. This will depend upon to what extent one of the latter fills a unit cell of the lattice. Figure 1A shows an intrinsic molecule, circular in cross section, occupying most of a unit cell. If we admit close packing of intrinsic molecules as $C \rightarrow 1$, then, if they are circular in cross section, they must occupy about 87% of the area of the unit cell. Molecules with somewhat irregular shape could occupy a greater percentage area. Here, for simplicity, we shall assume that the molecules are approximately circular. This is justified because we are only interested in establishing what is the *average* number of lipid molecules that interact with the intrinsic molecules over a time period of 10^{-3}–10^{-5} s (the characteristic ^2H NMR time scale). In this time even large proteins may undergo a considerable amount of rotational and translational diffusion (unless restricted by interactions with skeletal networks) so that the approximation of an effective circular cross-sectional area is reasonable.

Different Lipid Populations. We must next decide how many lipid molecules in one-half of a bilayer can (i) occupy a unit cell, (ii) fit around an isolated intrinsic molecule, (iii) be trapped between a pair of isolated molecules, and (iv) be trapped in addition to those of (iii) by three molecules arranged on an equilateral triangle. These numbers will be denoted by n_F, n_1, n_2, and n_3, respectively. In order to calculate the total number of lipid molecules, we must calculate the probability of such configurations arising as a function of C.

We shall assume that the relative cross-sectional areas of a lipid molecule and an intrinsic molecule are given by the ratio of their molecular weights, M_L and M_M. Thus

$$A_M = M_M A_L/(2M_L) \quad (1)$$

where A_L and A_M are the effective cross-sectional areas of a lipid molecule and an intrinsic molecule. There is a factor of 2 in the denominator of eq 1 because if the intrinsic molecule spans the bilayer we must associate a molecular weight per intrinsic molecule of $M_M/2$ with each half of the bilayer. The approximate number of lipid molecules that can be packed into a unit cell of the intrinsic molecule lattice (Figure 1A) is

$$n_F = M_M/(2M_L\alpha) \quad (2)$$

where α is the proportion of the unit cell that can be occupied by an intrinsic molecule. The radii of a lipid chain and an intrinsic molecule are given by $R_C = [A_L/(2\pi)]^{1/2}$ and $R_M = (A_M/\pi)^{1/2}$. By referring to Figure 1B we see that the approximate number of lipid chains that can fit around an isolated intrinsic molecule is

$$2n_1 = \pi(R_M + R_C)/R_C \quad (3)$$

Now, while each 60° sector of a unit cell (crosshatched in Figure 1A) can hold $n_F/6$ lipid molecules, this is only true if the site adjacent to the crosshatched region is not occupied by an intrinsic molecule. The reason is that, generally, $\alpha \gtrsim 3^{1/2}/2$ because we must allow for the possibility that intrinsic molecules can be in contact. Thus, if such a molecule is adjacent to an unoccupied unit cell, some of those lipid molecules adjacent to the molecule will encroach upon the area of the neighboring unit cell so that less than n_F lipid molecules, *which are not adjacent to an intrinsic molecule*, can be placed in the unoccupied unit cell. About half of the lipids in a 60° sector adjacent to an intrinsic molecule will "spill over" into a neighboring unit cell. By using the fact that the number of nearest-neighbor unoccupied unit cells is $N(1 - C)^2$, while the number of unoccupied unit cells having an unoccupied unit cell as a nearest neighbor is $NC(1 - C)$, we see that the approximate number of lipid molecules in an unoccupied unit cell, which are not adjacent to any intrinsic molecule, is

$$N_F = N(1 - C)[n_F - (n_1/2)C] \quad (4)$$

The approximate number of lipid molecules that are adjacent to an intrinsic molecule but not trapped, as described by (iii) or (iv), can be calculated as follows: Define a bond to be a straight line (in graph theory this is called an edge) connecting two lattice sites. It is a "dangling bond" if one of the sites is occupied by an intrinsic molecule. It is a "paired bond" if both sites are occupied by intrinsic molecules (Figure 1C). The total number of bonds in the lattice is $6NC$. The number of paired bonds is $3NC^2$; hence, the number of dangling bonds is $6NC - 2(3NC^2) = 6NC(1 - C)$, the factor of 2 entering in the second term because each paired bond removes two bonds from the dangling bond population. Each dangling bond has associated with it $\sim n_1/6$ lipid molecules adjacent to the intrinsic molecule so that the number of such lipid molecules is

$$N_A = Nn_1C(1 - C) \quad (5)$$

It then follows that the number of lipid molecules in categories iii and iv is respectively

$$N_{T2} = 3Nn_2C^2$$

$$N_{T3} = 2Nn_3C^3 \quad (6)$$

Equations 4–6 account for all the lipid molecules in one-half of a bilayer. The values of n_2 and n_3 will depend upon α, and they can only be estimated, principally because n_2 can only

be estimated from knowing the relative sizes of a lipid and an intrinsic molecule.

The total number of lipid molecules in one-half of a bilayer, N_L, is thus $N_L = N_F + N_A + N_{T2} + N_{T3}$. In order to calculate the ^2H NMR quadrupole splitting, $\Delta\nu_Q$, of a DMPC methyl group, we shall assume that all lipids in a given population, i–iv, exhibit the same splitting and that these are $\Delta\nu_F$, $\Delta\nu_A$, $\Delta\nu_{T2}$, and $\Delta\nu_{T3}$. Thus

$$\Delta\nu_Q = (N_F\Delta\nu_F + N_A\Delta\nu_A + N_{T2}\Delta\nu_{T2} + N_{T3}\Delta\nu_{T3})/N_L \quad (7)$$

if we assume that there is a fast exchange (compared to the characteristic ^2H NMR time scale) between lipids of different populations. Evidently, as $C \rightarrow 0$ all lipids belong to category i so that we shall choose $\Delta\nu_F$ to be equal to the observed splitting in a pure lipid bilayer. Also, as $C \rightarrow 1$ all lipids are in categories iii and iv, and we shall choose $\Delta\nu_{T2} = \Delta\nu_{T3}$ to be the splitting observed at very high concentrations of intrinsic molecules. We thus have two parameters, α and $\Delta\nu_A$.

Finally, the weight percent of the intrinsic molecule, W, and the intrinsic molecule/lipid weight ratio, P, are

$$W = 100C/[C + 2M_LN_L/(NM_M)]$$

$$P = NM_MC/(2N_LM_L) = W/(100 - W) \quad (8)$$

In the next section we shall choose α and $\Delta\nu_A$ in order to analyze the observed effect of gramicidin A′ upon the ^2H quadrupole splitting, $\Delta\nu_Q$, of the end methyl group of DMPC. On the grounds that the polypeptide gramicidin may approximate to a hydrophobic segment of an intrinsic protein, we shall then use the same value of $\Delta\nu_A$ to analyze the experimental observations on cytochrome oxidase.

Comparison of Experimental Data with Calculations. When we examine the published experimental data obtained by means of ^2H NMR spectroscopy on intrinsic protein–lipid and polypeptide–lipid systems, we find that there appear to be two situations occurring, one exemplified by gramicidin A′ and the other exemplified by large proteins such as cytochrome oxidase. Both these cases differ, in turn, from that of the effects due to the small intrinsic molecule cholesterol.

The quadrupole data for gramicidin A′ at 30 °C show a complicated behavior (Rice & Oldfield, 1979): up to ~16 wt % gramicidin the quadrupole splitting of the terminal methyl group, $\Delta\nu_Q$, of the lipid 2 chain increases to 4.5 from 3.4 kHz for pure DMPC. This then decreases to 3 kHz at ~40 wt % when a rapid decrease to 0 occurs by 50 wt %. In contrast to these results, cholesterol (Jacobs & Oldfield, 1979, Figure 7) causes $\Delta\nu_Q$ to increase monotonically at least up to cholesterol concentration $c = 0.4$ at 30 °C. For higher concentrations it may decrease slightly. Thus gramicidin A′ gives an ordering effect at low polypeptide concentrations and, at higher concentrations, a disordering effect on the lipid chains.

In order to understand these observations within the framework of different lipid populations, we calculate n_F, n_1, n_2, and n_3 and assign values to α, $\Delta\nu_F$, $\Delta\nu_A$, $\Delta\nu_{T2}$, and $\Delta\nu_{T3}$. There are only two parameters, α and $\Delta\nu_A$, and the value of α is constrained to lie between ~0.87 and <1.0. Figure 2 shows cross sections of gramicidin molecules and lipid chains in the plane of one-half of a bilayer. Since the DMPC bilayer is in a fluid phase, the area of the chain is $A_L/2 \simeq 30$ Å^2. If we assume that the areas scale like the molecular weights, then, taking that of gramicidin to be 1800, the area of a gramicidin molecule is ~159 Å^2, which is equivalent to 2.65 lipid molecules. If we assume a circular cross section for gramicidin, then $\alpha = 0.87$ so that ~3 lipid molecules can fit into a unit cell, or one chain in each 60° sector of the unit cell. This means that $n_F = 3$. It then follows that about nine chains can

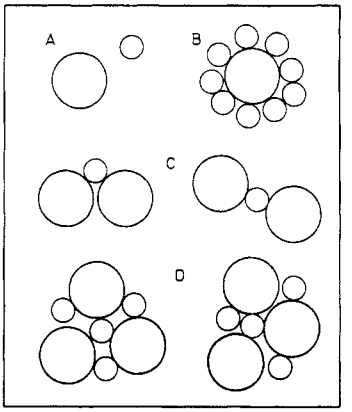

FIGURE 2: (A) An isolated gramicidin molecule and a lipid chain in population i. (B) An isolated gramicidin surrounded by nine chains in population ii. (C) A pair of gramicidin molecules with one chain trapped in population iii. (D) A triangle of gramicidin molecules with three chains trapped in population iii and one in population iv.

fit around an isolated gramicidin molecule (Figure 2B), so that $n_1 \simeq 4.5$. Figure 2C shows that it is reasonable to assume that about one lipid chain could be trapped between a pair of gramicidin molecules so that $n_2 \simeq 0.5$. Finally, Figure 2D shows that with this choice of n_2, about one more chain can be trapped by a triangle of gramicidin molecules so that $n_3 \simeq 0.5$. As we stated in the last section, $\Delta\nu_F$ is determined by the value of $\Delta\nu_Q$ for the methyl group at $C = 0$. Various values have been given [~3.4 kHz (Rice & Oldfield, 1979), 3.6 kHz (Rice et al., 1979a), and 3.7 ± 0.2 kHz (Kang et al., 1979)]. In this paper we chose a value of 3.6 kHz. In the case of trapped lipids it is physically reasonable to assume that the effect on the methyl group is the same whether trapped by two or by three intrinsic molecules so that we may choose $\Delta\nu_{T2} = \Delta\nu_{T3}$. Furthermore, as $C \rightarrow 1$ the gramicidin A′ structure becomes nearly close packed, so in this limit all the lipids are in trapped populations with the corresponding splittings. It is known (Rice et al., 1979a,b) that the methyl group splitting decreases monotonically for concentrations greater than ~20 wt % gramicidin and that it eventually goes to zero. With the assumption that, up to ~50 wt %, the DMPC–gramicidin A′ system is a bilayer, we must choose the splittings of the trapped lipids to be less than $\Delta\nu_F$ or $\Delta\nu_A$, and the simplest choice is $\Delta\nu_{T2} = \Delta\nu_{T3} = 0$. It might be noted that an alternative explanation is that the decrease in $\Delta\nu$ may be due to rapid lipid motion on the surface of a vesicle [see, e.g., Burnell et al. (1980)]. Here we are presenting the consequences of our model if it turns out that such an alternative explanation does not hold. We are then left with only $\Delta\nu_A$ to choose, the quadrupole splitting of methyl groups belonging to chains in population ii as shown in Figure 2B. It should be noted that although we have chosen the ^2H quadrupole splitting of the end methyl group of trapped lipids to be zero, as suggested by the results of Rice & Oldfield (1979), it is not essential that this value be used. A value sufficiently smaller than that of the splitting observed in pure lipids will account for the observed splitting in gramicidin A′. The same remark will, of course, hold for cytochrome oxidase (below).

We find that a value of $\Delta\nu_A = 6$ kHz gives a good fit to the data, and the plot of $\Delta\nu_Q$ together with the measurements of Rice & Oldfield (1979) is shown in Figure 3. Note that it is the value of $\alpha = 0.87$ that determined the weight percent of gramicidin at which the splitting decreases abruptly. It is, therefore, encouraging that a reasonable value of α yields agreement with observation. These results show that the methyl group of a chain adjacent to an isolated gramicidin molecule is somewhat more ordered than that of a chain in

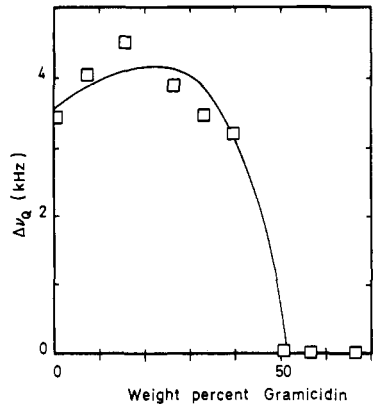

FIGURE 3: Calculated ^2H quadrupole splitting for a DMPC methyl group (solid line) as a function of wt % gramicidin A′: $\Delta\nu_F$ = 3.6 kHz, $\Delta\nu_A$ = 6 kHz, and $\Delta\nu_{T2} = \Delta\nu_{T3}$ = 0. □ indicates the data of Rice & Oldfield (1979) at 30 °C.

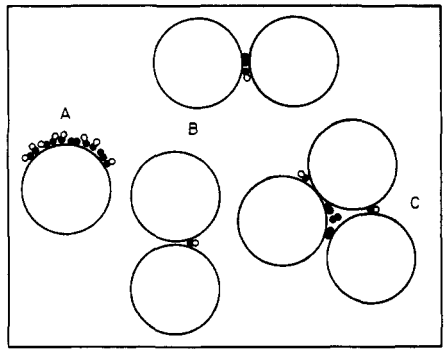

FIGURE 4: (A) An isolated cytochrome oxidase molecule showing lipid molecules adjacent to it. The dark areas are the chains in population ii. (B) A pair of cytochrome oxidase molecules showing lipid chains (dark areas) trapped in population iii. (C) A triangle of cytochrome oxidase molecules showing molecules trapped in populations iii and iv.

a pure DMPC bilayer at 30 °C. We shall leave the discussion of this to the next section.

When we consider the experimental data obtained for the large protein cytochrome oxidase, we see (Kang et al., 1979, Figure 7) that the ^2H quadrupole splitting, $\Delta\nu_Q$, of the lipid methyl group is approximately constant (or increases slightly) up to ~60 wt % protein. This is followed by an abrupt decrease to 2.6 kHz at 90 wt % protein. No splittings are published for higher weight percentages.

Again we must evaluate the parameters involved, and to do so we assume that the protein has a cross-sectional area given by eq 1. Taking the molecular weight to be ~160000, we find a cross-sectional area of ~7080 Å^2, which is equivalent to ~118 lipid molecules. For a reason that we shall mention below, we chose α = 0.94 (instead of 0.87). This means that cytochrome oxidase, because its cross section is not a circle, can pack more densely than gramicidin. This gives n_F = 125.5, and by using eq 3 we find that $n_1 \simeq 25.7$. Figure 4B shows that each pair of proteins can "trap" ~1 lipid molecule. By calculating the area available to lipids enclosed by a triangle of three proteins on nearest-neighbor sites and subtracting half of the lipids trapped by the three paired bonds, we find that a triangle of three such proteins can trap an extra 2.25 lipid molecules. Thus $n_2 \simeq 1$ and $n_3 \simeq 2.25$, and, as before, we choose $\Delta\nu_F$ = 3.6 kHz.

Having obtained the value of $\Delta\nu_A$ = 6 kHz from our analysis of gramicidin A′, we shall use this value, together with the values of $\Delta\nu_{T2} = \Delta\nu_{T3}$ = 0, to calculate the concentration dependence of the methyl group splitting, $\Delta\nu_Q$, for lipids in DMPC–cytochrome oxidase bilayers at 30 °C. Thus, we have

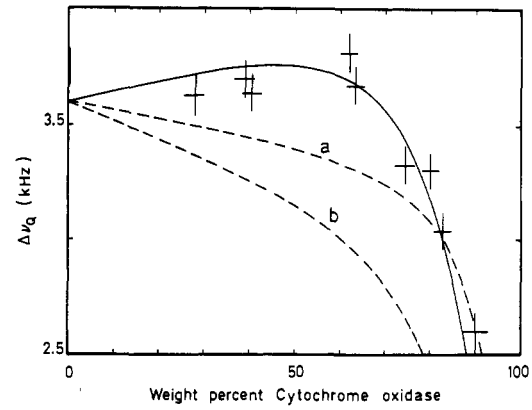

FIGURE 5: Calculated DMPC methyl group ^2H quadrupole splitting (solid line) as a function of wt % cytochrome oxidase with the same parameters used for gramicidin A′ ($\Delta\nu_F$ = 3.6 kHz, $\Delta\nu_A$ = 6 kHz, and $\Delta\nu_{T2} = \Delta\nu_{T3}$ = 0). + indicates the data of Kang et al. (1979) at 30 °C. The dashed lines are the predictions of calculations that assume that lipids adjacent to one protein (i.e., in category ii) are more disordered than pure lipid at the same temperature. (a) $\Delta\nu_F$ = 3.6 kHz and $\Delta\nu_A = \Delta\nu_{T2} = \Delta\nu_{T3}$ = 2.0 kHz; (b) $\Delta\nu_F$ = 3.6 kHz and $\Delta\nu_A = \Delta\nu_{T2} = \Delta\nu_{T3}$ = 0.

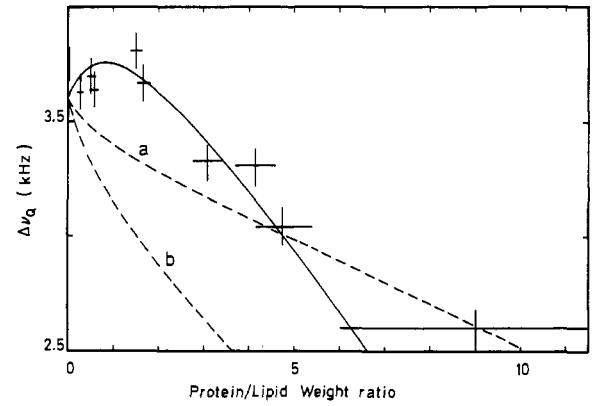

FIGURE 6: The same data calculations of Figure 5 plotted as a function of protein/lipid weight ratio. Note that the horizontal error bars of Figure 5 become larger on this plot than shown by Kang et al. (1979).

essentially no free parameters except α. This is chosen so that the decrease in $\Delta\nu_Q$ occurs at the correct weight percent of protein. Figures 5 and 6 show (solid lines) the ^2H quadrupole splitting of the methyl group as a function of weight percent protein and the protein/lipid weight ratio together with the data of Kang et al. (1979). Note that the horizontal error bars shown in Figure 5 become very wide for high protein/lipid weight ratios in Figure 6. The theoretical curves predict that there should be a slight increase in $\Delta\nu_Q$ over the concentration range from 0 to ~50 wt %. Note that, although we have reproduced the vertical error bars shown by Kang et al. (1979, Figure 7), that shown at 0 wt % protein is much smaller than the value of ±0.2 kHz quoted. With a value of 3.7 ● 0.2 kHz for the pure lipid, our value of 3.6 kHz would lie well within the range allowed. If the other points also have error bars of ±0.2 kHz, then our calculated curve will pass through all the points.

Other lipid populations can be considered. For example, instead of three populations there may be only two: those adjacent to proteins, trapped or otherwise, and others. We would then have $\Delta\nu_F$ = 3.6 kHz and $\Delta\nu_A = \Delta\nu_{T2} = \Delta\nu_{T3}$. The dashed curves in Figures 5 and 6 show the results of calculations based upon these lipid populations. Curve a has $\Delta\nu_A$ = 2 kHz, while curve b has $\Delta\nu_A$ = 0, and it can be seen that they differ qualitatively from the published experimental data. An alternative assumption that each protein has associated

with it an annulus of ~25.7 lipid molecules in each half of the bilayer and that this is disordered also does not reproduce the observed experimental data. The measurements on gramicidin A′ and cytochrome oxidase in DMPC bilayers at 30 °C thus appear to be in accord with a model that predicts fast exchange between three populations of lipids.

In the next section we shall discuss various points that have arisen, suggest a physical mechanism that might give rise to what we have proposed, and, on the basis of it, make some predictions.

Discussion and Conclusions

We have proposed a model for the effect of gramicidin A′ and cytochrome oxidase upon the static ordering of the terminal methyl group of DMPC at 30 °C in terms of various lipid populations, whose concentration varies with the concentration of the intrinsic protein in the lipid bilayer. The model proposes that for lipid chains adjacent to *one* intrinsic molecule, the methyl group is somewhat more ordered than that in a pure lipid bilayer at the same temperature ($\Delta\nu \simeq$ 6 kHz compared to a pure lipid splitting of ~3.6 kHz, both of which are very much less than the splitting, 38.4 kHz, of a rapidly rotating methyl group in a nonrotating all-trans chain) and that for lipid chains trapped between a cluster of two or three intrinsic molecules the methyl group quadrupole splitting is ~0 kHz, so that they are more disordered statically than a pure lipid. The model also proposes that there is complete exchange between these three populations of lipids on a time scale of 10^{-5} s. That the latter is possible can be seen by calculating the mean-square displacement $\overline{r^2}$ for lipids. The lateral diffusion coefficient, D, is related to $\overline{r^2}$ by $\overline{r^2} = Dt$, where t is the average time elapsed in traversing random paths which yield $\overline{r^2}$. Rubenstein et al. (1979) and Kuo & Wade (1979) have found that $D \gtrsim 2 \times 10^{-8}$ cm^2/s at 26 °C. Using this value of D and $t = 10^{-5}$ s, we find that $(\overline{r^2})^{1/2} \simeq 45$ Å. This is approximately the radius of a cytochrome oxidase protein so that 10^{-5} s is ample time to allow lipid molecules to exchange between the different populations. The same conclusion would obviously hold for the case of gramicidin A′.

On a time scale of ~10^{-8} s, characteristic of electron spin resonance (ESR) spectroscopy, however, we find that for $t = 10^{-8}$ s $(\overline{r^2})^{1/2} \simeq 1.5$ Å, which is far too small to allow exchange of lipids between the three populations. We have pointed out elsewhere (Hoffman et al., 1981; note that in this paper the concentration in Figure 2B should read 0.1 and 0.2) that the ESR data of Knowles et al. (1979) can be understood by a model that assumes that as long as one spin-labeled chain is adjacent to a protein then its motion will be sufficiently "mechanically" hindered, due to the presence of attached ESR probes, to display an "immobile" component. This would comprise all lipids in populations ii and iii. This calculation involved no parameters other than the protein concentration and the size of the protein. If, in addition, spin-labeled lipids in population iv are also mechanically hindered in this fashion, then two spectra of lipids should be observed, that from population i (free lipid) and that from populations ii–iv.

It should be clear that although we propose that intrinsic molecules can perturb lipids adjacent to them, as we have maintained elsewhere (Pink & Chapman, 1979; Pink & Zuckermann, 1980), there is no evidence that supports the concept of a long-lived unbroken annulus of lipid surrounding each intrinsic molecule. Indeed, our results can only be interpreted as showing that this cannot be the case.

We have also shown for the case of cytochrome oxidase (Figures 5 and 6) that chain disordering due to a rough protein surface alone is not sufficient to understand the published experimental results (Kang et al., 1979). It is evident that such a mechanism would be quite inadequate to understand the gramicidin A′ results.

An alternative explanation for the behavior of the quadrupole splittings in the presence of gramicidin has been proposed (Rice & Oldfield, 1979). It was suggested that lipid chains adjacent to a gramicidin molecule might be constrained in twisted configurations in the crevices of the molecule surface. It was then proposed that this gramicidin–lipid complex presents a smooth cholesterol-like surface to the remainder of the lipids, which it then orders. It is not explained, however, how such a short-lived complex, having a lifetime $<10^{-5}$ s and with which lipid chains are thus rapidly exchanging, can have such a smooth surface so as to make the other lipids more ordered than bulk lipid at temperatures above T_c.

Our interpretation of the dependence of $\Delta\nu_Q$ upon intrinsic molecule concentration in the fluid phase is that the observed behavior arises through a competition between the slight ordering due to the hydrophobic surface of the intrinsic molecule and the disordering that results when the lipids are trapped between proteins. The question remains then as to the physical reason for these effects.

Our findings of the tendency for an intrinsic molecule to order the terminal methyl groups adjacent only to it simply mean that although the hydrophobic surface is rough, it is not quite rough enough on the scale required to induce further disorder into the end of the chain. The disordering of the methyl groups in trapped lipid cannot then be understood as a consequence of the roughness of the hydrophobic regions of the intrinsic molecules, and a second competing effect is required.

We suggest that when intrinsic molecules form regions of high local concentration, they perturb the lipid polar layer so as to reduce the steric hindrance restricting the motion of the lipid hydrocarbon chain methyl group. If this mechanism is correct, then concentration effects in the polar region should occur.

If the decrease in the ^{2}H quadrupole splitting of the hydrocarbon chain methyl group arises because of effects occurring in the polar region of trapped lipids, then the concentration dependence of these effects can be calculated. Consider some quantity Q characteristic of a lipid polar group, and let it take on values Q_F, Q_A, or Q_T if the lipid molecule belongs to populations i, ii, or iii and iv, respectively. Q_F is thus the limiting value at low protein concentration while Q_T is the corresponding value at high concentrations. The average value of Q for any concentration of intrinsic molecule is then

$$Q = [Q_F N_F + Q_A N_A + Q_T(N_{T2} + N_{T3})]/N_L \qquad (9)$$

Our model predicts that $Q_A = Q_F$, and we have used eq 9 to calculate the concentration dependence of the full line width, 2δ, of ^{31}P NMR spectra of DMPC–cytochrome oxidase bilayers (Rajan et al., 1981). We chose $Q_F = Q_A = 80$ Hz and chose Q_T so that the calculated value of $Q = 2\delta$ coincided with the value of 300 Hz reported by Rajan et al. (1981) for 83 wt % cytochrome oxidase. We found that $Q_T = 935$ Hz. Figure 7 shows the experimental data of Rajan et al. together with our calculated curve (solid line), and the good general agreement lends support to our proposal that it is the polar groups of the trapped lipids that are perturbed by the proteins.

We have studied the alternative that all lipids adjacent to at least one protein, viz., those in populations ii–iv, have the line width changed by the same amount so that $Q_A = Q_T$. In Figure 7 the dashed lines show the results of these calculations. The parameter $Q_A = Q_T$ was chosen so that curve a passed

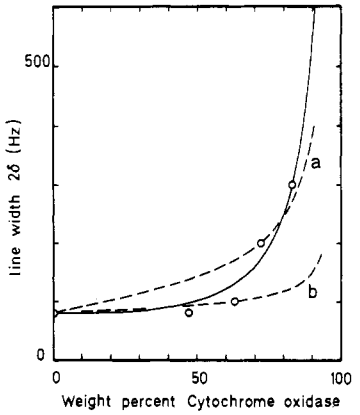

FIGURE 7: ^{31}P full line width, 2δ, for DMPC–cytochrome oxidase bilayers as a function of wt % protein at 30 °C. Circles indicate the data of Rajan et al. (1981). The solid line is that calculated from eq 9 with $Q_F = Q_A = 80$ Hz and $Q_T = 935$ Hz. The dashed lines are for $Q_F = 80$ Hz and (a) $Q_A = Q_T = 580$ Hz and (b) $Q_A = Q_T = 192$ Hz.

through the experimental point at 72 wt % protein, while curve b was adjusted to pass through the point at 63 wt % protein. It can be seen that these curves are qualitatively different from the trend in 2δ, thus lending further support to our proposal.

Some remarks should be made concerning the utility of plotting observations vs. protein/lipid weight ratio. As can be seen from a comparison of Figure 5 and Figure 6, the predictions of different models are all essentially linear for protein/lipid weight ratio $\gtrsim 1$ in the case of cytochrome oxidase. Such a plot thus provides little information about whether it is lipid methyl groups adjacent to proteins (population ii) or those trapped between proteins (populations iii and iv) that are being disordered more than those of pure lipid bilayers. It is the behavior of $\Delta\nu_Q$ in the region of low protein/lipid weight ratio that distinguishes the various mechanisms.

Finally, we studied over what range $\Delta\nu_A$ might be varied for the DMPC–cytochrome oxidase bilayer and still yield results in accord with experiment (Figure 5). We found that $\Delta\nu_A$ must lie between ~ 5 and ~ 7 kHz with a best fit, by eye, obtained by using $\Delta\nu_A = 6$ kHz. This appears to indicate that the hydrophobic surface of cytochrome oxidase has a similar effect, as to the static ordering of the methyl group, as does gramicidin A′. The reason why the splitting rises to over 4 kHz in the case of gramicidin A′, but only to ~ 3.7 kHz in the case of cytochrome oxidase, is the difference in sizes of the two molecules: the smaller size of gramicidin allows more lipids to be in the adjacent population than does cytochrome oxidase.

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