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EFFECT OF ALTERATIONS IN LIPID PACKING ORDER BY HYDROPHOBIC SOLUTES ON THE ASSOCIATION STATE OF PROTEIN ASSEMBLIES IN MODEL MEMBRANES

Keith J. STELZER and Michael A. GORDON *

Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.

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The effects of two hydrophobic solutes which perturb lipid packing order, permethrin and allethrin, on the aggregated state of a lipid membrane-incorporated protein, bacteriorhodopsin (BR), have been determined by resonance energy transfer measurements. As temperature is increased from well below the main gel-fluid phase transition temperature (T_c) of the lipid, patches of aggregated BR dissociate into monomers, a few degrees below the T_c (M.P. Heyn, A. Blume, M. Rehorek and N.A. Dencher, Biochemistry 20 (1981) 7109; M.P. Heyn, R.J. Cherry and N.A. Dencher, Biochemistry 20 (1981) 840).Permethrin and allethrin were found to cause a decrease in the temperature of BR disaggregation which was associated with a decrease in the T_c of the lipid. In gel phase dipalmitoylphosphatidylcholine at 25°C, the pertubing effects of permethrin on lipid packing order were associated with a decrease in the average patch radius from 123 to 33 Å. It is concluded that perturbation of lipid packing order by small hydrophobic molecules may alter the stability of protein assemblies in membranes.

1. Introduction

Protein-protein association in membranes is considered to be important in functional regulation. An important factor in the stability of protein assemblies may be interactions of the oligomers with surrounding membrane lipids. Accordingly, the packing order of the surrounding (solvation) lipid environment would be expected to contribute to the stability of protein assemblies [4]. Therefore, factors which perturb the packing order of solvation lipid may, as a consequence, alter the stability and dynamics of protein assembly.

For example, thermal disordering of solvation lipid surrounding a membrane protein, bacteriorhodopsin (BR), results in dissociation of the assembled protein [1,2]. Another example of membrane lipid disordering is that which results from the intercalation of small hydrophobic solutes into solvation and bulk lipid domains. However, it has not previously been determined if perturbation of lipid packing order by hydrophobic molecules can alter the stability of protein assemblies in membranes. Therefore, this study investigates the effects of compounds which disorder lipid packing on the association dynamics of a protein in model membrane systems.

Two pyrethroids, permethrin and allethrin, have been utilized in this study as examples of hydrophobic molecules which perturb lipid packing order. These compounds were selected because their lipid disordering effects have been well characterized by fluorometric techniques in cellular membranes [5], bulk lipid phases of model membranes [6–8], and boundary lipid domains surrounding polypeptides in model membranes (unpublished results). Furthermore, these compounds alter the function of several membrane proteins, such as the nicotinic acetylcholine receptor/channel [9,10] and Ca^{2+}-ATPase [11]. Interestingly, Ca^{2+}-ATPase [12] and the nicotinic acetylcholine receptor/channel [13] are proteins for which ag-

* To whom correspondence should be addressed.

gregation state may have functional implications.

In this study, BR reconstituted into phosphatidylcholine vesicles was used as a model for protein-protein association in a lipid membrane. At temperatures well below the gel-fluid phase transition temperature (T_c) of the lipid, BR aggregates into patches containing this protein at a high concentration [1,2,14]. As the temperature is increased to approx. 5°C below the T_c of the lipid, the patches of BR will disaggregate into monomers [1,2]. Using nonradiative fluorescence energy transfer from an excited fluorescent donor molecule to the retinal acceptor contained within Br, the size of patches containing aggregated BR may be approximated [3,15].

A principle of resonance energy transfer measurements is that the fluorescence intensity of donor molecules will be quenched by proximal acceptor molecules which absorb in the wavelength range of the donor emission spectrum. When patches are formed, only those BR molecules in the exterior portion of the patches are available to absorb energy [3]. A fraction of the BR molecules will be buried in the interior of patches. These BR molecules located in the patch interior are not in close proximity to the energy donor. Given the distance dependency of energy transfer efficiency, the result will be a lower amount of energy transfer than that which would have been measured if all the BR molecules were in the monomeric state, and as a consequence, close to donor molecules.

If the surface density of BR in the membrane is known, it is possible to obtain an estimate of how much BR is buried within patches (a function of patch size) by quantitative analysis of energy transfer data [3,15]. The temperature of patch disaggregation can also be determined by resonance energy transfer techniques [3]. Utilizing this approach, the effects of pyrethroid-induced solvation lipid perturbation on protein association in membranes may be evaluated.

2. Materials and methods

2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), BR (from *Halobacterium halobium*), and octylglucoside were purchased from Sigma (St. Louis, MO). Diphenylhexatriene (DPH) was purchased from Molecular Probes (Junction City, OR). Permethrin was generously donated by FMC Corp. (Princeton, NJ), and allethrin was a gift from McLaughlin Gormley King (Minneapolis, MN).

2.2. Lipid vesicle preparation

DPPC and DMPC were each dissolved in chloroform to a concentration of 20 mg/ml. DPH was dissolved in chloroform to a concentration of 0.2 mM. The solvated DPH was added to either DPPC or DMPC such that the lipid/DPH ratio was 500 : 1. The chloroform was evaporated under nitrogen, and the dried lipid was hydrated with 0.01 M phosphate-buffered saline (pH 7.4). The hydrated lipid was then warmed to approx. 15°C above the T_c of the lipid, vortex-mixed until the lipid film was resuspended, and sonicated at a micro-tip speed setting of 2.5 for 90 s with a model W140D sonicator from Heat Systems-Ultrasonics, (Plainview, NY).

Octylglucoside was dissolved in 25 mM phosphate buffer (pH 6.9) to a concentration of 1.15%. BR was dissolved to a concentration of 2.5×10^{-5} M in the octylglucoside solution in a polystyrene test tube. The tube was placed in a 50 ml beaker containing water at room temperature, and the surrounding water was sonicated for 20 s at a micro-tip speed setting of 5. The BR solution was then allowed to incubate in the dark at room temperature for 24 h prior to use. Following this 24 h incubation, the BR solution was stored at 5°C. The BR was not used for energy transfer measurements after 48 h had elapsed since dissolution. Prior to energy transfer measurements, the octylglucoside concentration was reduced in both donor and donor-acceptor samples by a 120-fold dilution with the phosphate buffer solution.

An aliquot, containing 7×10^{-7} mol DPPC or DMPC, of the sonicated vesicle suspension was added to an appropriate volume of the BR solution on ice. For determination of the temperature of BR disaggregation, the lipid/BR ratio was 300 : 1. For determination of BR patch radius in gel phase DPPC, the DPPC/BR ratio was varied

from 375 : 1 to 175 : 1. After incubation for 1 h on ice in the dark, the vesicle suspension was diluted in phosphate-buffered saline at 5°C to a final concentration of 6×10^{-5} M.

In determining resonance energy transfer, two samples were prepared. One of these samples (d-a) contained both the donor molecule (DPH) and the acceptor molecule (BR) in the vesicles. The other sample (d) contained the donor and bleached (non-absorbing) acceptor. This d sample was necessary as a control to determine the amount of fluorescence intensity of DPH in the absence of energy transfer. The d sample was also used in the determination of the lipid gel-fluid phase transition profile by measurement of DPH anisotropy, allowing BR to be present in the membrane without causing artificially high values of DPH anisotropy due to fluorescence quenching. BR, solvated in octylglucoside and placed in a glass test tube, was bleached by incubation for 1 h in a water-filled 600 ml glass beaker at room temperature, 35 cm from the center of a 450 W xenon arc lamp. The bleached BR was reconstituted into the lipid vesicles in the same manner as the unbleached BR.

2.3. Fluorescence measurements

For all fluorescence measurements, temperature was regulated by a peripheral circulating water bath coupled with water-jacketed sample chambers. The temperature was monitored by a digital thermometer. For experiments in which temperature was varied, an ascending temperature scale was used which increased at a rate which did not exceed 0.5°C/min. Pyrethroids were dissolved in dimethyl sulfoxide (DMSO) prior to addition to the vesicle samples to give a final concentration of 1×10^{-5} M. Control samples received an equivalent volume of DMSO (less than 0.1%, v/v).

Resonance energy transfer efficiencies were determined either from corrected fluorescence emission intensity measurements or from lifetimes calculated by phase-modulation techniques [16] using an SLM 4800 fluorometer. For corrected emission intensity measurements, incident light was set by monochromater to a wavelength of 360 nm. Light emitted from the sample chamber was passed through a Schott KV-389 cut-on filter. For correction of possible light source intensity fluctuations, emission from a reference sample of rhodamine was measured after passing the beam through a Schott RG-630 cut-on filter. Ratiometric fluorescence intensity measurements were alternately made for the d and d-a samples, and the average of 20 consecutive measurements was used to calculate efficiency of energy transfer.

Fluorescence lifetimes were calculated from phase shift and demodulation parameters as previously described [5]. The SLM 4800 fluorometer was configured as described above. Incident light was modulated in its intensity at 30, 18 and 6 MHz. A sample of 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene dissolved in ethanol was used as a standard of known lifetime (1.45 ns) for the calculation of fluorescence lifetimes. The average of four consecutive lifetime measurements was used to calculate energy transfer efficiency.

Fluorescence anisotropy measurements were made with an SLM 8000 fluorometer, configured in the T format with polarizers inserted in the excitation and emission beams. DPH was excited by incident light set at 360 nm. The incident light was alternately polarized in the horizontal and verticle orientations. Emitted light was measured at 426 nm utilizing an SLM MC320 monochromator placed in the vertically polarized emission beam. Horizontally polarized emission was measured by passing the beam through a Schott KV-389 cut-on filter.

2.4. Calculation of energy transfer efficiency and patch size

From the measurements of fluorescence intensity (I) and lifetimes (T) in the d-a and d samples, the ratios $I_{d\text{-}a}/I_d$ and $T_{d\text{-}a}/T_d$ were determined. The efficiency of energy transfer was calculated from these ratios as $(1 - I_{d\text{-}a}/I_d)$ and $(1 - T_{d\text{-}a}/T_d)$.

$I_{d\text{-}a}/I_d$ was also used to determine the average size of BR patches in gel phase DPPC at 25°C, using the previously described theory of resonance energy transfer to a circular patch of acceptors [3,15]. In this theory, approximants (A) are calculated from moments (M) to give the best fit to the measured value of $I_{d\text{-}a}/I_d$. The first two moments

are calculated as

$$M_1 = \pi^2 D R_0^6 (r+h)^2 r^2 / \left[S_A h^3 (2r+h)^3 \right] \quad (1)$$

$$M_2 = \pi^3 D D_P R_0^{12} r^4 \left[(9r^4/7) + (4r^3 h) + (26r^2 h^2/5) + (16rh^3/5) + (4h^4/5) \right] / \left[2S_A h^7 (2r+h)^7 \right] \quad (2)$$

in which D is the surface density of BR molecules in the vesicle, D_P the surface density of BR in a patch, R_0 the Forster distance (the distance between donor and acceptor at which 50% energy transfer efficiency is obtained), r the distance from the center of the patch to the retinal moieties of the BR molecules on the outer edge of the patch, h the distance of closest approach of DPH to the retinal of the BR, and S_A the surface area of the acceptor patch [3]. The surface density of BR, D, is estimated from the surface area of a BR molecule (875 Å^2) and the surface area of a phospholipid (60 Å^2) by the equation,

$$D = \left[875\ \text{Å}^2 + (60\ \text{Å}^2) C_L / (2C_{BR}) \right]^{-1} \quad (3)$$

where C_L and C_{BR} are the molar concentrations of lipid and BR, respectively [3]. The factor of 2 in the denominator of eq. 3 corrects for the fact that the lipid is distributed within two monolayers, whereas each BR protein spans the bilayer. D_P is similarly calculated from the surface areas of BR and lipid, considering that there are approx. 10 tightly bound lipids per BR molecule in a patch [1], by the equation,

$$D_P = \left[875\ \text{Å}^2 + (60\ \text{Å}^2)(10/2) \right]^{-1} \quad (4)$$

R_0 is calculated as

$$R_0 = \left(JK^2 Q_d n^{-4} \right)^{1/6} (9.7 \times 10^3\ \text{Å}) \quad (5)$$

in which J is the spectral overlap integral determined by the donor emission and the acceptor absorbance spectra [17], K^2 the characteristic orientational factor representing the relative orientation of the transition dipoles of the donor and acceptor (taken as 2/3 for rapid relative motion), n the refractive index of the media (1.33), and Q_d the quantum yield of the donor [3]. An extinction coefficient of 54600 M^{-1} cm^{-1} at 560 nm for BR [3] was used in the calculation of J. Absorption spectra used in the determination of J and Q_d were measured by an SLM/Aminco DW-2C spectrophotometer. Corrected fluorescence emission spectra of DPH used to calculate J and Q_d were measured with the SLM 8000 fluorometer configured for ratiometric intensity determination. The emission spectra of DPH were determined with bleached BR incorporated into the vesicles, either with or without permethrin, and at appropriate temperatures in order to ensure that the R_0 obtained was representative of the conditions under which the energy transfer measurements were made.

The spectral overlap integral, J, was calculated by measuring the absorption spectrum of BR and the emission spectrum of DPH in the membranes under experimental conditions. The spectral amplitudes were measured in arbitrary units (cm) at wavelength intervals of 50 Å. At each wavelength (w) the products $AFw^4 dw$ and $F dw$ were obtained, in which A is the amplitude of the absorbance spectrum (cm) at wavelength w, F the amplitude of the emission spectrum (cm) at wavelength w, and dw the wavelength interval (50 Å). In these products, w is expressed as Å. Using the extinction coefficient of BR given above along with the above products summated at 50 Å intervals, J is calculated from

$$J = \left[\Sigma AFw^4 dw \right] / \left[\Sigma F dw \right] \times 54\,600 / A_{560} \times 10^{-32} \quad (5)$$

The factor of 10^{-32} converts the units from cm^4 to Å^4 (C.A. Hasselbacher, personal communication).

The quantum yield of the donor, Q_d, was calculated using a standard consisting of 7.42×10^{-7} M quinine sulfate in 0.1 N H_2SO_4, which has a quantum yield (Q_s) of 0.55 at an excitation wavelength of 366 nm [18]. The corrected fluorescence emission spectra of the donor and the standard were measured using the same photomultiplier tube voltage settings for both scans. The spectra were integrated to give IF_d and IF_s for the donor and standard, respectively. The absorbances of the donor sample (A_d) and the standard (A_s) were also measured, using the same wavelength as that used to excite the samples for the fluorescence

scans (360 nm for DPH and 366 nm for quinine). The quantum yield of the donor was then calculated for each experimental condition by the equation,

$$Q_d = Q_s(IF_d/IF_s)(A_s/A_d) \quad (6)$$

The distance of closest approach, h, of DPH to the retinal of BR has been determined to be 18 Å [19]. The actual radius of the patch will be $r + 15$ Å. The additional 15 Å is the distance from the retinal of the outer layer of BR to the first layer of surrounding lipid, and is the distance h projected in the plane of the bilayer [3]. The surface area of the patch, S_A, is calculated as $(r + 15\ \text{Å})^2$.

From the moments, the approximants are calculated as

$$A_1 = (1 + M_1)^{-1} \quad (7)$$

$$A_2 = 1 - [M_1^2/(M_1 + M_2)] \quad (8)$$

A fitting program was employed to vary r systematically in order to obtain the value of $(A_1 + A_2)/2$ closest to $I_{d\text{-}a}/I_d$. The patch radius was then determined at $r + 15$ Å.

3. Results

The effects of the pyrethroids, permethrin and allethrin, on the the main gel-fluid lipid phase transition and on the disaggregation of BR patches are shown in fig. 1. As measured by DPH anisotropy, permethrin and allethrin were observed to decrease the T_c of DMPC and DPPC vesicles which contained bleached BR. These results are consistent with those reported in pure DPPC large multilamellar vesicles [6]. The dissociation of BR patches into monomers was detectable by an increase in the energy transfer efficiency calculated from the corrected fluorescence intensity (I). This process has been previously demonstrated [3]. As observed by other investigators [1,2], dissociation of BR patches occurred several degrees below the T_c of the lipids. The pyrethroids decreased the temperature of BR dissociation by a similar magnitude to that by which they decreased the T_c of the lipids.

From fig. 1, it is also apparent that the efficiency of energy transfer was attenuated at higher temperatures, temperatures at which BR is in the monomeric state, in the presence of pyrethroids. This attenuation of energy transfer efficiency was probably due to lower values of R_0 in the presence of pyrethroids. In DPPC at 45°C, for example, the values of R_0 were determined to be 42.6 Å in the control and 38.6 Å in the permethrin-treated sample. Lower values of R_0 decrease energy transfer efficiency.

Fig. 2 illustrates the temperature-dependent energy transfer efficiency profiles, calculated from

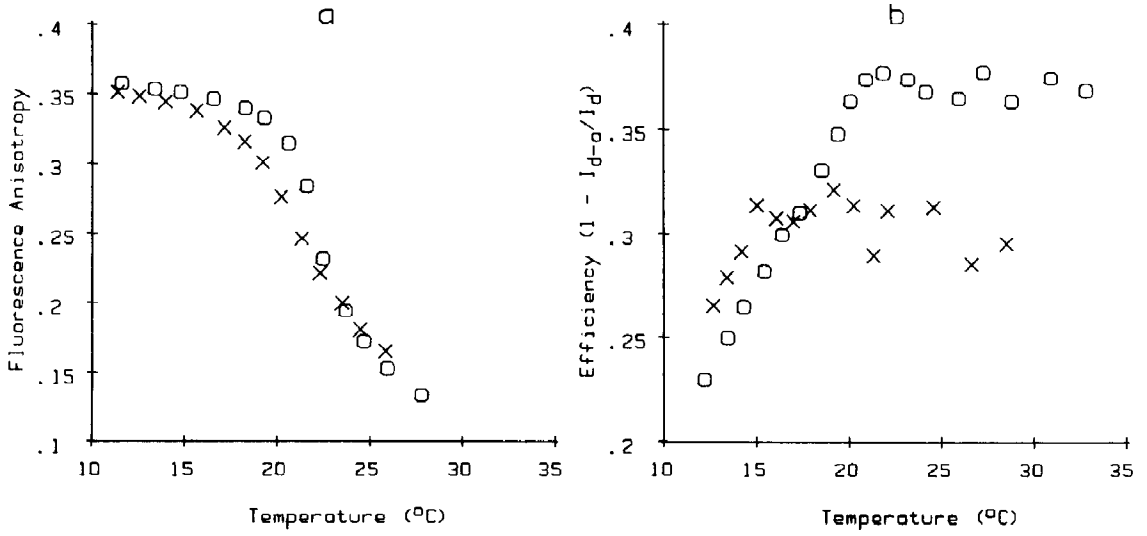

Fig. 1(a,b).

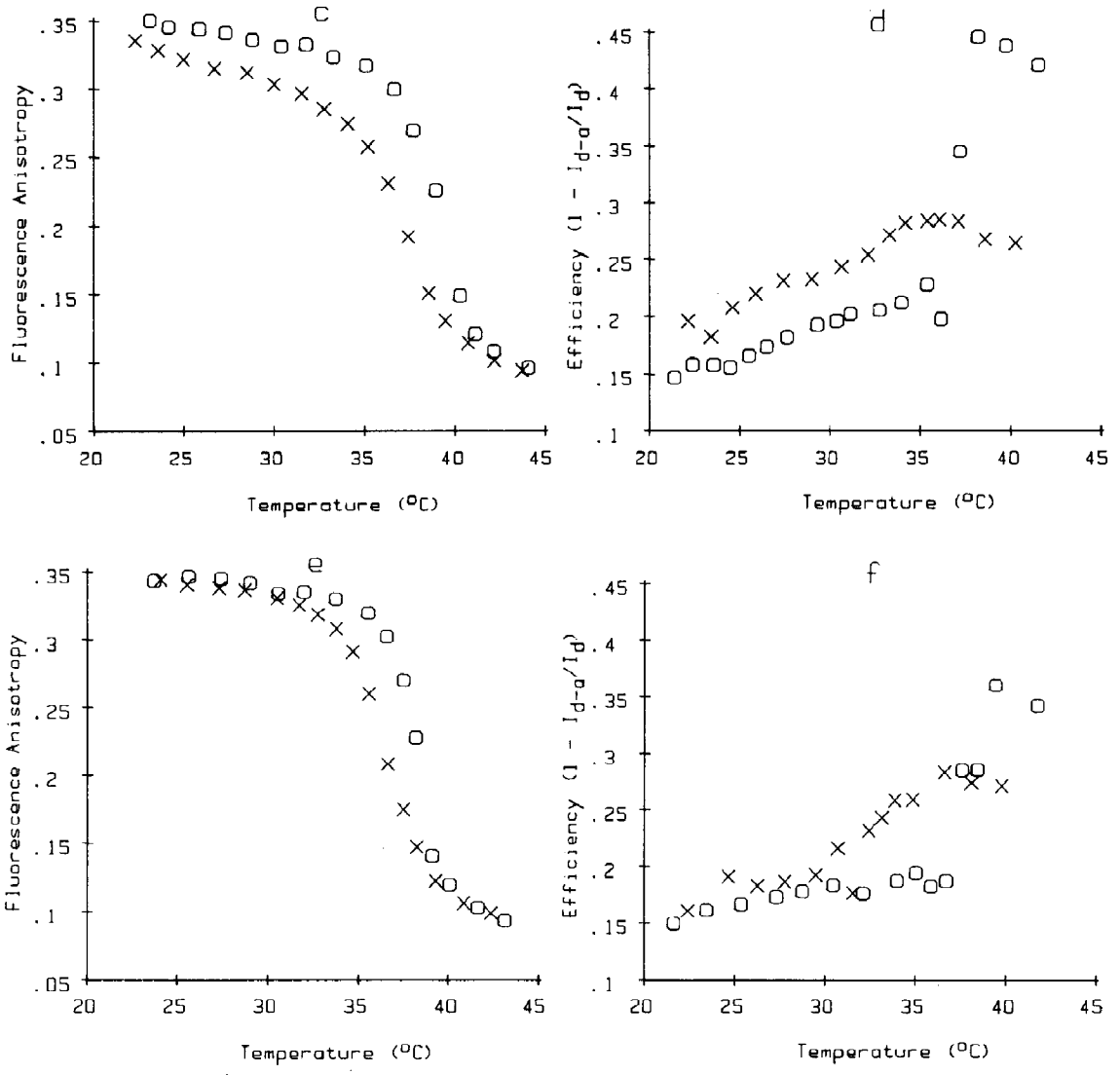

Fig. 1. Effects of pyrethroids (1×10^{-5} M) on the phase transition profile of phosphatidylcholine vesicles containing BR as determined by DPH fluorescence anisotropy, and on the dissociation of BR patches in phosphatidylcholine vesicles as determined by energy transfer efficiency calculated from fluorescence intensity (I). Effects of permethrin on: (a) anisotropy in DMPC/BR, energy transfer efficiency in DMPC/BR, (c) anisotropy in DPPC/BR, (d) energy transfer efficiency in DPPC/BR. (e) Effects of allethrin on: (e) anisotropy in DPPC/BR, energy transfer efficiency in DPPC/BR. (○) Control, (×) permethrin or allethrin.

the fluorescence lifetime (T), in the DPPC vesicles for the control and permethrin-treated samples. The lifetimes used to determine these profiles were calculated from phase shift. The modulation frequency was 30 MHz. As observed in fig. 1d, the temperature of BR patch disaggregation was decreased by permethrin. However, the efficiencies of energy transfer calculated by fluorescence lifetime are lower than those calculated from fluorescence intensity. A possible explanation is that much of the DPH will be highly quenched, resulting in a very short fluorescence lifetime. The

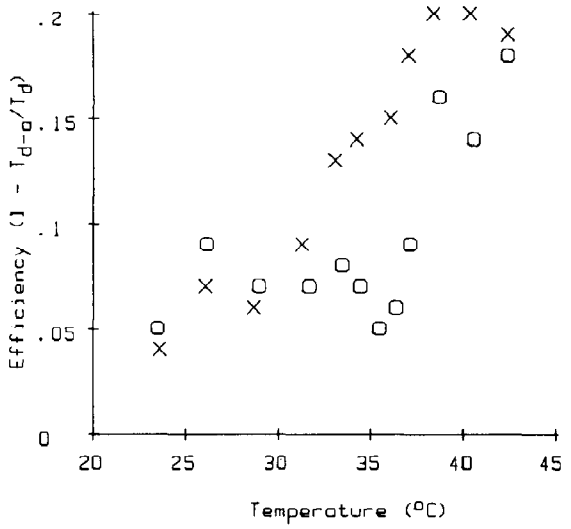

Fig. 2. Effects of permethrin (1×10^{-5} M) on BR patch dissociation in DPPC vesicles as determined by energy transfer efficiency calculated from fluorescence lifetimes (T). Fluorescence lifetimes were calculated from phase shift using a modulation frequency of 30 MHz. (O) Control, (×) permethrin.

quenching will be detected by a decrease in the steady-state fluorescence intensity. However, using phase-modulation lifetime techniques at the available modulation frequencies, the very short fluorescence lifetimes will not be readily detected. Supporting this explanation is the observation that the efficiencies of energy transfer were calculated to be lower using lifetimes determined from lower modulation frequencies and by demodulation instead of phase shift. Lifetimes calculated from these lower modulation frequencies and from demodulation are weighted to express longer lifetime components in a heterogeneous system [16], and are expected to be less sensitive to the highly quenched DPH than would be the lifetimes calculated from phase shift at a modulation frequency of 30 MHz. An additional factor may be a long-lifetime component of unknown origin which was present in the samples containing unbleached BR. This long-lifetime component was detected to a greater extent by lifetimes calculated from demodulation, particularly at lower modulation frequencies.

Another difference between the profiles shown in figs. 1d and 2 was the failure of permethrin to attenuate the energy transfer efficiency calculated from lifetimes with BR in the monomeric state. As noted earlier, permethrin shortens R_0 in fluid phase DPPC, and this shorter R_0 will cause a decrease in the energy transfer efficiency as measured by fluorescence intensity. However, since the phase modulation lifetime measurements appear to be relatively insensitive to the highly quenched population of DPH, it is expected that the effect of R_0 on energy transfer efficiencies calculated from phase-modulation lifetimes should be diminished.

The effects of permethrin on $I_{d\text{-}a}/I_d$ as a function of BR surface density in gel phase DPPC vesicles at 25°C are illustrated in fig. 3. Vesicles were prepared such that the minimum value of surface density was 8×10^{-5} Å^{-2}. At surface densities greater than this value, a constant patch size was observed which was characteristic of the particular lipid and temperature [3]. Lower surface densities result in breaking up of BR patches into smaller units [3]. Fig. 3 shows that permethrin causes lower values of $I_{d\text{-}a}/I_d$ (higher values of energy transfer efficiency). These data, in consideration with the lower value of R_0 in the presence

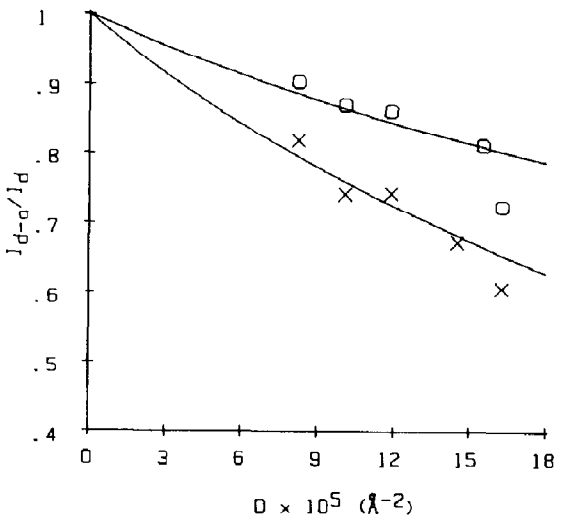

Fig.3. Effects of permethrin (1×10^{-5} M) on $I_{d\text{-}a}/I_d$ as a function of BR surface density in DPPC vesicles at 25°C. (O) Control, (×) permethrin. The theoretical curves are based upon the assumption of constant patch size, and are calculated by the approximant theory [3,15].

of permethrin, indicate that permethrin causes patch dissociation in gel phase DPPC at 25°C. Patch radii were calculated to be 123 ± 30 Å for the control and 33 ± 7 Å for the permethrin-treated sample. It has been estimated [1] that each BR molecule in a protein patch is surrounded by 10 lipids (5 per monolayer). From the surface area of BR and the phospholipid, it can be estimated that permethrin decreased the patch size from approx. 40 proteins/patch, in the control sample, to approx. 3 proteins/patch.

4. Discussion

The disaggregation, with increasing temperature, of BR patches into monomers has been attributed to the partial melting of a solvation layer of lipids ('solvation lipids') which surround the BR molecules in a patch [1]. By differential scanning calorimetry, the melting of these solvation lipids can be detected to occur several degrees below the gel-lipid phase transition temperature of the bulk lipid [1]. In the present study, pyrethroids decreased the T_c of DMPC and DPPC in the form of sonicated vesicles containing bleached BR. The results were consistent with previous work in pure DPPC large multilamellar vesicles, which also demonstrated a decrease in the bulk lipid phase transition temperature in response to pyrethroid treatment [6]. The pyrethroids were also found to decrease the temperature of BR patch dissociation to monomers. From these results, pyrethroids appear to decrease the melting temperature of the protein solvation lipids in much the same way as these compounds decrease the melting temperature of the bulk lipid phase.

Phase-modulation lifetime measurements confirmed the lower temperature of BR disaggregation in permethrin-treated DPPC vesicles. However, the energy transfer efficiencies calculated from the lifetime measurements were not as high as those calculated from fluorescence intensity measurements. The low values of energy transfer efficiency calculated from lifetime measurements are indicative of the high degree of quenching of donor fluorescence for those DPH molecules in close proximity to BR molecules. This high degree of donor fluorescence quenching should be expected, considering that the distance of closest approach, 18 Å [19], of DPH to the retinal portion of BR is well within the values of R_0 (approx. 40 Å) calculated for this system. Lifetimes measured by fluorescence decay following flashlamp excitation of DPH gave efficiencies of energy transfer which were in good agreement with efficiencies obtained by fluorescence intensity measurements [19]. The flashlamp technique [19] could detect donors which were quenched to a greater extent than those detectable by phase-modulation techniques at a modulation frequency of 30 MHz.

Fig. 1c shows that permethrin disordered gel phase lipid packing in DPPC/BR vesicles. This disordering was detected by a decrease in DPH anisotropy. To determine if the lipid disordering was associated with decreased BR patch size in the gel phase lipid, $I_{d\text{-}a}/I_d$ was analyzed in DPPC containing a range of BR surface densities at 25°C. It was found that permethrin decreased the number of BR molecules aggregated in a patch from approx. 40, in the control, to approx. 3. Therefore, the solvation lipid is similar to the bulk lipid phase with respect to its sensitivity to the lipid disordering properties of permethrin. Under the conditions of this experiment, permethrin was able to cause enough disordering of the solvation lipid to reduce patches to trimeric structures. Further disordering of the solvation lipid by thermal means resulted in monomer formation, as indicated by the small rise in energy transfer efficiency upon disaggregation in the presence of permethrin (fig. 1d). Although the trimeric patch does not contain a population of BR molecules which are inaccessible for energy transfer by burial in the center of the patch, the trimeric BR molecules do have a reduced overall surface area which is available for close alignment by DPH molecules.

Pyrethroids, which have been shown to perturb lipid packing order in cell membranes [5], in the bulk lipid domain of model membranes [6–8], and in the boundary lipid domain surrounding polypeptides incorporated into model membranes (unpublished results), have now been shown to cause dissociation of protein assemblies within a membrane. To our knowledge, this is the first demonstration that hydrophobic molecules which alter

lipid packing order can also alter protein association dynamics. The results suggest that disordering of solvation lipid domains caused by intercalation of hydrophobic solutes destabilizes protein assemblies.

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