PAIR DISTRIBUTION FUNCTIONS OF BACTERIORHODOPSIN AND RHODOPSIN IN MODEL BILAYERS

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ABSTRACT The pair distribution functions have been measured from freeze-fracture pictures of bacteriorhodopsin and rhodopsin recombinants with diacyl phosphatidylcholines (PC) of various hydrocarbon chain lengths. Pictures were used of samples that had been frozen from above the phase transition temperature of the lipid. Measured functions were compared with those calculated from two model interparticle potential energy functions, (*a*) a hard-disk repulsion only, and (*b*) a hard-disk repulsion plus electrostatic repulsion for a point charge buried in the membrane. The measured functions for bacteriorhodopsin di 12:0 PC, di 14:0 PC, and di 16:0 PC recombinants can be simulated using an interparticle hard-disk repulsion only. Bleached rhodopsin di 12:0 PC and di 18:1 *trans*-PC recombinants, and dark-adapted rhodopsin di 10:0 PC recombinants yield functions that are better simulated by assuming an additional repulsive interaction. The measured functions resemble those calculated using the hard-disk plus electrostatic repulsion model. The picture of dark-adapted rhodopsin in di 18:1 *trans*-PC frozen from 20°C shows partial aggregation that is apparent in the measured pair distribution function. This attractive interaction persists even at 37°C, where the measured function shows deviations from the hard-disk repulsive model, indicative of an attractive interparticle interaction. Implications of these results are discussed in terms of protein-lipid interactions.

INTRODUCTION

The aggregation state of proteins in biological membranes is an important aspect of membrane organization. It has been shown that the lateral organization of membrane bound proteins can change during events of physiological significance (for example, Decker and Friend, 1974; Staehelin, 1976; Friend and Rudolf, 1974; Weinstein, 1976). The most direct method for determining the aggregation state of membrane proteins is freeze-fracture electron microscopy, which yields qualitative information upon simple visual inspection. Recently methods have been devised to quantitate the distribution of particles in micrographs, either by digitization of particle coordinates or using less elaborate counting techniques (Donnell and Finegold, 1981; Pearson et al., 1979).

In the present work we employ the pair distribution function (Perelson, 1978; Gershon et al., 1979; Markovics et al., 1974) to obtain a quantitative measure of the aggregation state of two transmembrane proteins, bacteriorhodopsin (BR) and rhodopsin (RH) in vesicles made with phosphatidylcholines (PC) of several acyl chain lengths. From the measured functions one can deduce the strength of protein-protein interactions, even if these interactions are too weak to produce aggregation that is visually obvious. Of particular interest is the possibility of lipid-mediated interactions (Marcelja, 1976; Marcelja et al., 1977), currently a subject without much data.

Spectroscopic experiments indicate that protein molecules perturb lipid order, and these data have been interpreted in terms of a perturbed boundary around the protein. Although it is possible that the boundary is a single layer of molecules, there is mounting data to suggest that it extends further (Marsh et al., 1982). In the latter case, there will be a characteristic length associated with decay of the lipid state to its bulk configuration, and the perturbation around the protein-lipid boundary would be expected to decay smoothly into the bulk lipid.

One way in which proteins could affect neighboring lipids is by altering the local membrane thickness. There are two ways that this thickness can be modified. The intramolecular conformational state of the hydrocarbon chains or the head groups can be affected, or tilting of entire lipid molecules may occur. For example, if the

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thickness of the hydrocarbon region of a membrane is less than that of the hydrophobic region of a protein, in order for the membrane to accommodate the protein, the lipid hydrocarbon chains would need to be in a more extended conformation with a higher proportion of *trans*-isomers per hydrocarbon chain than in the bulk lipid. The situation is somewhat more complex if the membrane hydrophobic region has to contract to accommodate the protein. In this case accommodation could take place either by an increase in the average tilt of the lipid chains with respect to the bilayer axis, or by further disordering of the chains via an increase in the fraction of gauche isomers along the hydrocarbon chain. It is apparent that for there to be a match between bilayer and protein, there will be a boundary region interfacing the protein surface with the bulk lipid.

If the proteins perturb the lipid to any significant extent, over a few nanometers laterally in the membrane, this lipid-protein interaction should have observable effects on the protein pair distribution function (Pearson and Chan, 1982). The pair distribution function, $w(r, \Delta r)$ is defined by the ratio of the mean density of particles located in an annulus between r and $r + \Delta r$ from the center of an average particle, to the mean overall density. A more fundamental quantity is the radial correlation function, $g(r)$, which is related to the pair distribution function by

$$w(r, \Delta r) = \frac{2\int_r^{r+\Delta r} g(r) r dr}{\Delta r(2r + \Delta r)}. \quad (1)$$

Although the pair distribution function, $w(r, \Delta r)$, approaches $g(r)$ as Δr approaches zero, for the relatively small (<1000) number of particles generally seen in freeze-fracture micrographs, only the pair distribution function can be determined reliably (using a finite annulus size to minimize relative errors in the determination of the number of particles in the annulus). Despite this shortcoming, the radial correlation function for a set of particles remains useful in that it can be readily calculated from a given interparticle potential energy function between the protein particles (Lado, 1968).

We illustrate this by considering here a number of special situations. If the protein particles were randomly diffusing points of infinitesimal size, it can be shown that $g(r)$, and hence $w(r, \Delta r)$ would be identically 1.0. If the particles were instead hard disks of radius r_o, then $g(r)$ would assume more of a structure (Lado, 1968), which at sufficiently high overall particle density would consist of a peak at $r = 2r_o$ corresponding to a first coordination shell, followed by a region of lower density at $r \approx 4r_o$. This pair distribution function can be calculated from the potential energy function

$$V(r) = 0 \qquad r \geq 2r_o$$
$$V(r) = \infty \qquad r < 2r_o. \quad (2)$$

The usefulness of this hard-disk model is that deviations of actually observed $g(r)$ or $w(r, \Delta r)$ from this result could be used to indicate additional attractive or repulsive interparticle interactions.

It should be emphasized that implicit in the above relationships is the assumption that the radial correlation function, and hence the interparticle potential energy function, is radially symmetric. If there is an additional angular correlation between particle positions, Eq. 1 for $w(r, \Delta r)$ must be written in terms of a more general function including angular and radial correlations. We defer a full discussion of this point until a later paper, but note at this juncture that the magnitude and shape of the pair distribution function $w(r, \Delta r)$ yields information about radial correlations that can be related directly to the form of interparticle interactions.

MATERIALS AND METHODS

BR-Lipid Recombinants

The preparation of BR-lipid recombinants is described in detail by B. A. Lewis and D. M. Engelman (manuscript submitted for publication) and is summarized below. BR was separated from its native lipid by the method of Huang et al. (1980). All operations were performed in the dark at 0–4°C. PC were from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA) and were lyophilized and dissolved in 2% Na cholate buffer (0.01-M Tris HCl, 0.15-M NaCl, pH 8) with brief sonication. To prepare BR-lipid recombinants, the lipid and BR solutions were mixed under dim red light and subsequently dialyzed against 0.01-M Tris HCl, 0.15-M NaCl, pH 8 in a foil-covered flask at 4°C. After at least three days of dialysis, the vesicles were centrifuged at 104,000 *g* through continuous sucrose gradients, 10–45% (wt/wt). All preparations yielded a single band. The vesicle bands were washed two or three times in buffer, 0.01-M Na acetate, pH 5.

For electron microscopy the BR-lipid recombinants were spray frozen by the method of Bachmann and Schmitt (1971) using a spray freeze apparatus (Balzers, Hudson, NH) with a thermally controlled spray gun. Fracturing and replication were performed in a freeze-etch unit (BAF 301; Balzers) at temperatures of −115 to −135°C and pressures of 1 to 8×10^{-7} torr. Deposition of Pt and C from electron beam electrodes was controlled by a quartz crystal monitor to a thickness of 25 and 200 Å, respectively. Replicas were examined in an electron microscope (EM) at 80 kV (EM 201; Philips Electronic Instruments, Inc., Mahwah, NJ) and at magnifications of 20,000 to 40,000 times. Photographs from the microscope were enlarged to 150,000–200,000 times for measurement of pair distribution functions.

RH-Lipid Recombinants

EM pictures of dark-adapted and bleached RH were taken from Chen and Hubbell (1973). The pictures used were of samples that had been frozen from above the phase transition temperature of the lipid. For dark-adapted RH the pictures chosen were of those recombinants in di 10:0 PC frozen from 20°C, and di 18:1 *trans*-PC frozen from 37 and 20°C and for bleached RH recombinants in di 12:0 PC and di 18:1 *trans*-PC frozen from 20°C.

Protein Distribution Functions

Protein pair distribution functions were determined by measuring particle coordinates from the EM pictures, calculating the interparticle distances and counting the number of interparticle distances that lie within the

annulus r to $r + \Delta r$. Particle coordinates were taken on the Tektronix graphics tablet (Tektronix, Inc., Beaverton, OR) in Dr. Jean-Paul Revel's laboratory at the California Institute of Technology and processed on a VAX 11/780 computer (Digital Equipment Corp., Marlboro, MA). Measured distribution functions were compensated for picture-edge effects by simulating random distributions in a square of the same area as that of the membrane analyzed. Up to 10 simulations were analyzed and the average pair distribution function taken as a baseline for the membrane measurements. Changing the shape of the test area from a square to a rectangle had negligible effect on the baseline. A value of Δr = 10 nm was used in calculating $w(r, \Delta r)$. This choice produced a reasonably noise-free distribution function while retaining the major details of the first peak in the function. The number of particles (N) in the picture was required to be greater than ~500 to minimize artifacts due to noise. Theoretical hard-disk pair distribution functions were obtained using (*a*) the potential energy function of Eq. 2, and (*b*) a potential energy function that includes both a hard-disk core and repulsive electrostatic interaction between two charges buried in the hydrophobic region of the membrane, namely (Tsien and Hladky, 1982),

$$V(r) = \infty \qquad r < 2r_0$$
$$V(r) = \frac{2\mu^2}{\epsilon\epsilon_0 r^3} \qquad r \geq 2r_0, \tag{3}$$

where ϵ is the relative dielectric constant of the lipid; ϵ_0 is the permittivity of free space; $\mu = q\ell$, where q is the magnitude of the charge, and ℓ is the depth that the charge is buried in the membrane. The correlation function, $g(r)$, was calculated using the Percus-Yevick and Ornstein-Zernike equations in the algorithm of Lado (1968). The Ornstein-Zernike equation (Eq. 4) permits us to relate the indirect correlation function, $h(r)$, of a system of particles to the direct correlation function, $c(r)$.

$$h(r) = g(r) - 1 = c(r) + \rho \int c(|r - s|)\, h(s) \mathrm{d}^2 s. \tag{4}$$

At the densities used in this study the direct correlation function is well approximated by the Percus-Yevick relation (Eq. 5)

$$c(r) = g(r)\,\{1 - \exp[V(r)/kT]\}. \tag{5}$$

We solved Eqs. 4 and 5 iteratively using the numerical algorithm of Lado (1968). An initial guess of $g(r)$ was used in Eq. 5 to calculate $c(r)$. The Hankel transform of $c(r)$, namely $\mathbf{c}(k) = \int_0^\infty c(r) J_0(kr) r \mathrm{d}r$, was then calculated and used in Eq. 6 to calculate the Hankel transform $\mathbf{h}(k)$, to obtain a second approximation to $h(r)$.

$$\mathbf{h}(k) = \frac{\mathbf{c}(k)}{1 - \rho \mathbf{c}(k)}. \tag{6}$$

The second approximation to $g(r)$, given by $h(r) + 1$, was then obtained by inverting $\mathbf{h}(k)$ and used in Eq. 5 to reiterate on $c(r)$. This process of calculating $\mathbf{h}(k)$ from Eq. 6 and using the inverse in Eq. 5 is repeated until convergence. Finally, the radial correlation function calculated from this algorithm was fitted to a polynomial and then integrated according to Eq. 1 to give $w(r, \Delta r)$.

RESULTS

Fig. 1 shows micrographs of BR reconstituted into di 12:0 PC, di 14:0 PC, and di 16:0 PC. Areas of membrane that showed even shadowing were chosen to minimize artifacts due to membrane curvature. There appeared to be no gross protein aggregation due to the freezing process in any of the pictures we examined. Rearrangement of the protein particles, in particular, a tendency to aggregate, should be reflected in the pair distribution function as an apparent attractive interaction between particles.

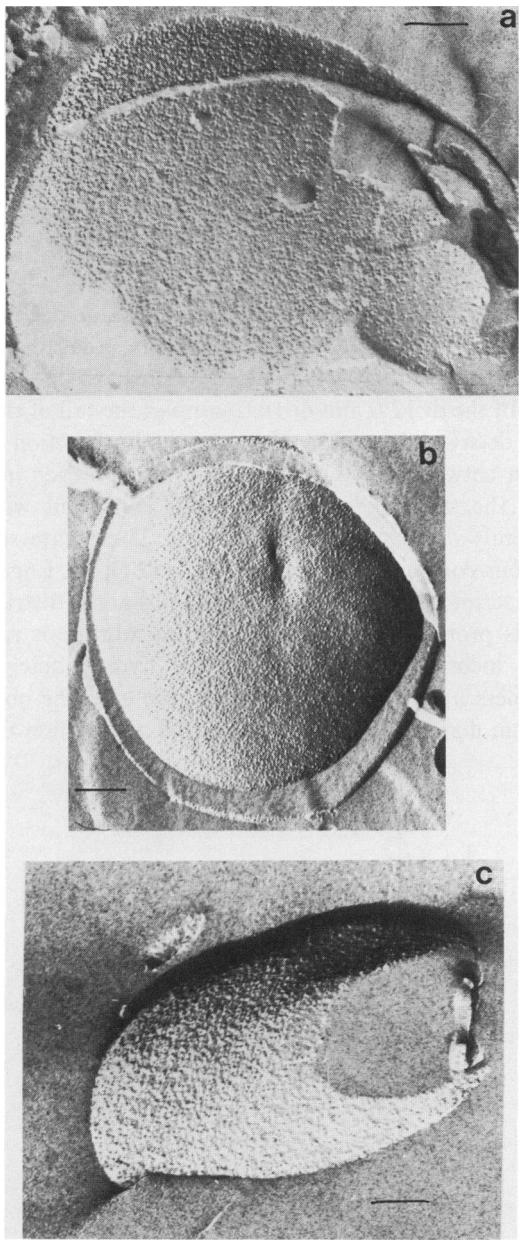

FIGURE 1 Representative micrographs of BR-PC recombinants. (*a*) di 12:0 PC, (*b*) di 14:0 PC, and (*c*) di 16:0 PC. The bars represent 150 nm.

The most significant characteristics of the measured functions for the purposes of comparison with the calculated functions are the magnitude of the function at the peak and its rate of decay toward the asymptotic value as $r \rightarrow \infty$. Interparticle interaction should be reflected in these features of the pair distribution function. In particular, an attractive interaction between particles would result in the peak being larger and decaying more slowly than the hard-disk model. For each of the measured functions presented in Figs. 2, 3, and 5, we show for comparison

(dashed line) the corresponding function calculated for the hard-disk model for the corresponding density and particle radius. The error bar shown on each measured function represents $\pm 1/\sqrt{n}$, where n is the number of interparticle distances that were used to calculate the value of that function at $r = 2r_0$. We now consider the results obtained for each protein in turn.

Bacteriorhodopsin

The measured functions are shown in Fig. 2 *a–c*. Comparison with the calculated functions indicates that in all three lipids, the protein is well modeled by the hard-disk interaction. In the di 12:0 and di 14:0 samples the tail of the first peak decays less rapidly than the model function in the region between $r = 8.0$ to 14.0 nm, though even in these cases the magnitude of the peak is consistent with the randomly diffusing hard-disk model. These data support previous conclusions (Lewis, B. A., and D. M. Engelman, manuscript submitted for publication) that the distribution of this protein in the plane of the membrane is random when incorporated into bilayers of hydrophobic region thickness up to 10 Å less than the protein. The observed random distributions also support the assumption that no significant aggregation of protein particles has occurred during the freezing process.

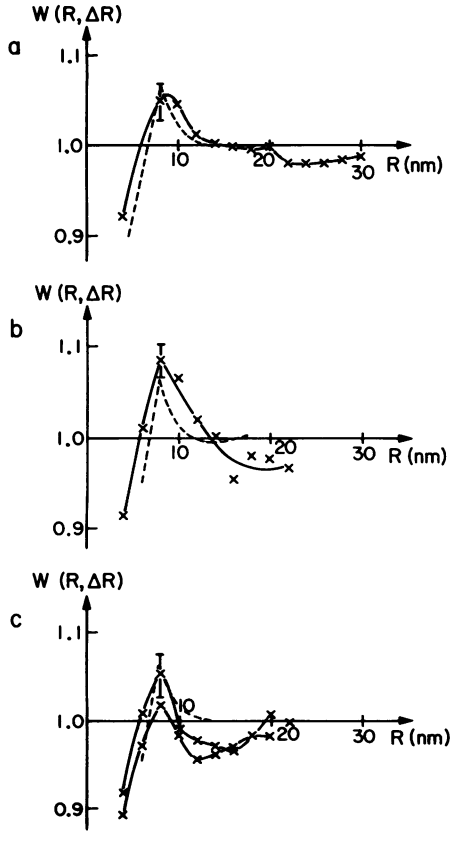

FIGURE 2 (*a*) Measured function for BR in di 12:0 PC. Number of particles $N = 1{,}022$, $\rho = 0.0054$ nm^{-2}. Frozen from 22°C. (*b*) Measured function for BR in di 14:0 PC. $N = 1{,}678$, $\rho = 0.004$ nm^{-2}. Frozen from 34°C. (*c*) Measured functions for BR in di 16:0 PC. $N = 1{,}099$ and 524, $\rho = 0.003$ and 0.0035 nm^{-2}, respectively. Frozen from 50°C.

Bleached Rhodopsin

The measured functions are shown in Fig. 3 *a* and *b*. The functions are more difficult to interpret, particularly as they do not seem to decay to 1.0 as r increases beyond the first peak, as in the case of the BR-PC recombinants. This suggests that Eq. 1 may not be holding exactly for these systems possibly because the radial correlation function is no longer radially symmetric. This lack of radial symmetry is seen clearly in certain of the RH pictures where there appear to be patches of lipid ringed by particles. Despite this complication, the difference in magnitude between the first peak and the asymptotic value of $w(r, \Delta r)$ should still be representative of interparticle interactions. In particular, the magnitude of the first peak can still be compared with that of the hard-disk model to ascertain whether there are additional interactions.

In both di 12:0 PC and di 18:1 *trans*-PC, the size of the first peak in the bleached RH pair distribution functions is less than that of the calculated function using the hard-disk model. In general, a value of $g(r)$, and hence $w(r, \Delta r)$, less than that calculated for a system of hard disks suggests that there is a longer range repulsive interparticle interaction tending to keep particles apart. This repulsion may be mediated, for example, by a charge on the protein buried some distance into the hydrophobic region of the membrane. In Fig. 4 we show pair distribution functions

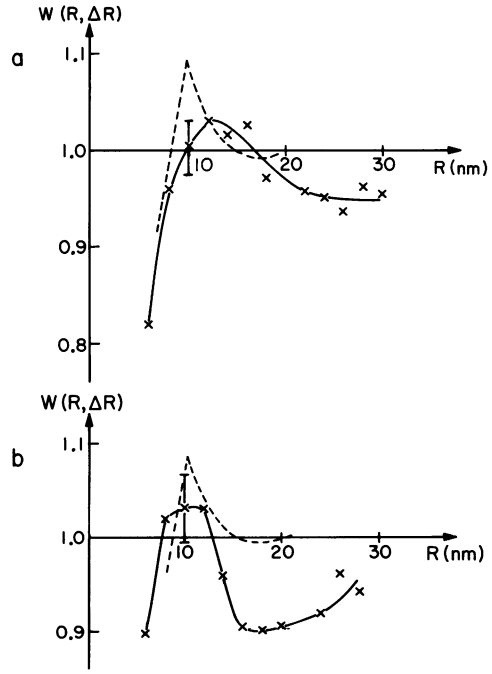

FIGURE 3 (*a*) Measured function for bleached RH in di 12:0 PC from Fig. 1 of Chen and Hubbell (1973). Frozen from 20°C. $N = 1{,}106$, $\rho = 0.0021$ nm^{-2}. (*b*) Measured function for bleached RH in di 18:1 *trans*-PC, Fig. 5 of Chen and Hubbell. $N = 668$, $\rho = 0.0017$ nm^{-2}.

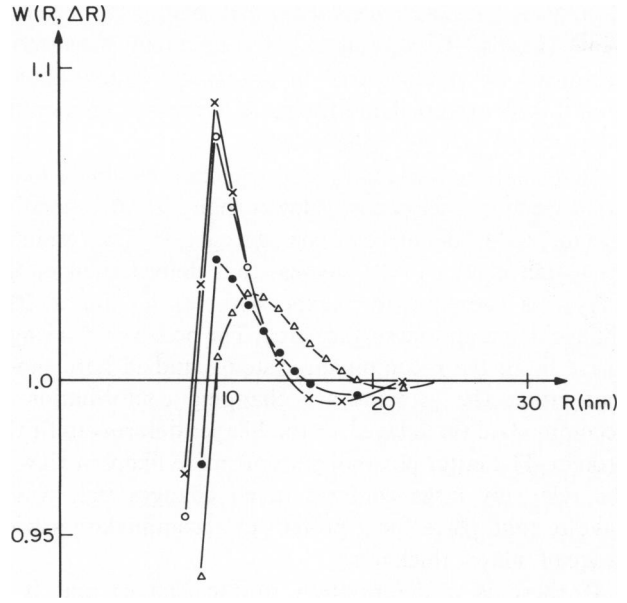

FIGURE 4 Calculated functions using the potential energy function of Eq. 3. $r = 5.0$, $\rho = 0.002$ nm^{-2}. ×, $\mu^2 = 1.0$; O, $\mu^2 = 2.5$; ●, $\mu^2 = 10.0$; Δ, $\mu^2 = 16.0$.

calculated for a system of particles for which each particle carries a charge q buried at a depth ℓ in the membrane (q is in electronic charge units), and these pair distribution functions are calculated for $\mu^2 = 1.0$, 2.5, 10.0, and 16.0. r_0 is 5.0 nm and $\rho = 0.002$ nm^{-2}. ϵ is taken to be 2.1. Comparison with the measured functions of Fig. 3 indicates that the size and shape of the measured functions are better modeled by an ionic repulsion of this type than by just a hard-disk repulsion alone. A value of μ of the order of 3 to 4 charge-nanometers appears to reproduce the data.

Dark-adapted Rhodopsin

The EM picture of dark-adapted RH in di 18:1 *trans*-PC frozen from 20°C (Fig. 4 *a* of Chen and Hubbell, 1973) shows the protein to be obviously highly aggregated although the aggregates are dispersed throughout the lipid phase. We chose to examine the picture of the di 18:1 *trans*-RH sample frozen from 37°C to ascertain whether the correlation persists at the higher temperature. Fig. 5 *a* and *b* show the measured pair distribution functions deduced from the data of Chen and Hubbell (1973) for dark-adapted RH-di 10:0 PC, and di 18:1 *trans*-PC recombinants frozen from 20 and 37°C, respectively.

The magnitude of the peak in the pair distribution function obtained from the 10:0 RH recombinant is consistent with the functions measured from the bleached RH pictures. In the di 18:1 *trans*–PC-RH sample frozen from 37°C, however, the peak is significantly larger and the rate of decay of the function from its peak is slower than the random hard-disk model. These features of the pair distribution function suggest that in dark-adapted RH-di 18:1 recombinants there is an attractive interaction between

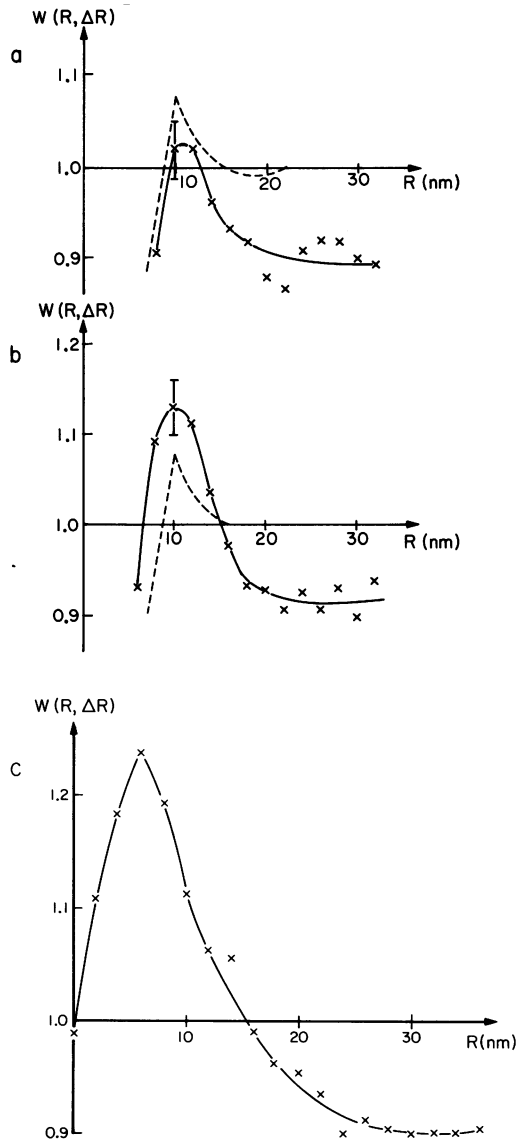

FIGURE 5 (*a*) Measured function for dark-adapted RH in di 10:0 PC. From Fig. 1 of Chen and Hubbell (1973). Frozen from 20°C. $N = 819$, $\rho = 0.0021$ nm^{-2}. (*b*) Measured function for dark-adapted RH in di 18:1 *trans*-PC. From Fig. 4 of Chen and Hubbell (1973). Frozen from 37°C. $N = 955$, $\rho = 0.0014$ nm^{-2}. (*c*) Measured function for dark-adapted RH in di 18:1 *trans*-PC. From Fig. 4 of Chen and Hubbell (1973). Frozen from 20°C. $N = 669$, $\rho = 0.0045$ nm^{-2}.

protein particles. Since this interaction does not exist in the thinner bilayer, it is therefore probably lipid mediated. These observations lend credence to ideas of lipid-mediated interactions proposed previously (Marcelja, 1976; Pearson and Chan, 1982).

In Fig. 5 *c* we show for comparison the pair distribution function deduced from the EM picture of dark-adapted RH in di 18:1 frozen from 20°C reported by Chen and Hubbell (1973). Here the protein is visibly aggregated. Aggregation is manifested as a relatively large value at the peak in $w(r, \Delta r)$. In addition, the peak occurs at a lower value of r than in the other measured functions for RH-PC systems. This appears to be a general effect that we have

noted in all measurements taken from pictures of partially aggregated systems. We defer a full discussion of this phenomenon until a later paper.

DISCUSSION

We have examined in this study the pair distribution functions in thin bilayers of BR, bleached RH, and dark-adapted RH. There appears to be negligible difference between the measured functions for the BR-PC systems and those calculated from a model in which the only interaction is a hard repulsive core. The value of $2r_0$ measured from the positions of the peaks in the pair distribution function is ~8 nm, which is in accordance with the diameter of the particles seen in the freeze-fracture micrographs. Although this figure is approximately twice that deduced by Peters and Cherry (1982) from measurements of the lateral diffusion coefficient of the protein in di 14:0 PC, it is consistent with the size of the BR trimer (Stamatoff et al., 1982), the state of aggregation often implicated for this protein. It is also likely that a freeze-fracture particle of BR represents a protein molecule with one or two boundary layers of lipid relatively tightly bound.

The bleached RH-di 12:0 PC and di 18:1 *trans*-PC and dark-adapted RH-di 10:0 PC systems show deviations from the hard-disk model that may be due to longer range interparticle repulsions and can be modeled assuming an interparticle interactions due to a buried charge or charges in the membrane. For dark-adapted RH-di 18:1 *trans*-PC recombinants, however, the results are strikingly different. The freeze-fracture micrograph from Chen and Hubbell (1973) of a sample frozen from 20°C shows visible aggregation. We have shown that even at 37°C, where the aggregation is not readily apparent from the EM picture, the protein distribution is not equivalent to that in which there is only a hard, repulsive core interaction between molecules, but that there appears to be an additional attractive component in the interaction.

These results can be understood in terms of the probable behavior of the lipids at the protein-lipid boundary and their effect or lack of effect on subsequent lipid solvation layers. When a protein is reconstituted into a bilayer for which the equilibrium thickness of the hydrophobic region does not match that of the protein, there are two possible consequences. The molecules may remain undeformed, thereby exposing the protein hydrophobic region to the aqueous phase in the case where the protein is thicker than the bilayer, or exposing the hydrophilic region of the protein to the hydrocarbon region of the bilayer if the protein is thinner. Alternatively, the bilayers or the proteins may deform at some energetic cost (Parsegian et al., 1979) to match their respective hydrophobic regions. Estimates of the energy cost of exposure of the protein hydrophobic surface to water suggest that if insufficient deformation occurs to match the hydrophobic regions, aggregation of the protein molecules will take place. Such aggregation has been observed in BR-di 10:0 PC recombinants (Lewis, B. A., and D. M. Engelman, manuscript submitted for publication). In this case the acyl chains, even if fully extended, are probably too short to match the hydrophobic surface of the protein.

Deformation of the bilayer or protein is the more likely event when its energy cost is lower than the corresponding cost due to loss of entropy upon aggregation. The results of Parsegian et al. (1979) suggest that deformation of the bilayer is energetically inexpensive, <1 kT for a 20% change in membrane surface area. The presence of a single phase in all the recombinant systems studied here shows that either the proteins are changing conformation to accommodate the bilayer, or the bilayer deforms to fit the protein. The latter possibility seems more likely in view of the relatively large conformational changes that would have to take place for a protein to accommodate a wide range of bilayer thicknesses.

If there is a deformation to the bilayer and it is propagated over a significant characteristic length in the membrane, an intermolecular potential energy will result (Pearson and Chan, 1982), which would be seen in the measured pair distribution functions. This is not seen in any of the systems studied here in which the membrane is thinner than the protein. An interaction is seen, however, in dark-adapted RH-di 18:1 *trans*-PC recombinants, in which the membranes are significantly thicker than those of other lipids used although not, it should be noted, particularly thick relative to most natural bilayer membranes.

To postulate an explanation for this effect we consider the cases of thick and thin membranes in turn. Consider first the case where the bilayer is thinner than the protein. If the bilayer deforms to fit the protein, stretching of the hydrocarbon region of the bilayer must occur at the protein-lipid boundary. Our results suggest that in the system we study here, the intermolecular conformational change that leads to this stretching is not propagated to any extent laterally through the membrane.

The observation of an interaction in dark-adapted RH-di 18:1 *trans*-PC recombinants suggests that the nature of the effect that this protein has on di 18:1 *trans*-PC bilayers is fundamentally different from that in the other systems examined. If the hydrophobic region of the bilayer formed by di 18:1 PC were thicker than that of the dark-adapted RH molecule, then the lipid molecules could tilt at the protein-lipid boundary, or there could be a conformational change in the lipid head group or hydrocarbon chain region. In any case, the change in the bilayer structure must be propagated over a significant distance in the membrane for an interprotein interaction to result. This change could involve tilting of the lipid molecules that may possibly be propagated through the bilayer by low-frequency cooperative motions of the hydrocarbon chains, or propagation of the direct lipid-protein interaction may be via the hydrophilic or interface regions of the lipid, or

merely increased disorder of the lipid-hydrocarbon chains. Further studies are needed to characterize the altered bilayer conformation.

The different behavior noted here between bleached and dark-adapted RH in di 18:1 *trans*-PC deserves comment. It is possible that the hydrophobic surface of RH thickens on bleaching so that the bleached molecule is no longer thinner than the di 18:1 *trans*-bilayer. Such a conformational change could reduce the perturbation of the bilayer by the protein and thus reduce the lipid-mediated interprotein attraction. This suggests the possibility of a role for lipid-protein interactions in the physiological function of RH, which should be investigated further (Baldwin and Hubbell, 1982). However, other explanations for the dramatic difference in aggregation of the two states of RH cannot be ruled out without further data. For instance, bleaching of RH might increase the net molecular charge, leading to an intermolecular repulsive interaction that could counteract lipid-mediated or other attractions. The fact that bleached RH remains unaggregated below the lipid-phase transition temperature in all lipids examined, under conditions in which dark-adapted RH aggregates, is more consistent with the latter explanation. Again, more experiments are required to establish the nature of the difference between dark-adapted and bleached RH in di 18:1 *trans*-bilayers.

There are other factors that may affect the observed protein distribution of the RH-lipid recombinants that are difficult to exclude completely at this time. First, pictures of the RH-PC recombinants were of multilamellar dispersions. It is possible that interbilayer interactions may affect lateral proteins. Similarly, in a multilamellar protein-lipid recombinant, the orientation of the protein incorporated into the recombinant membranes may influence the lateral distribution of membrane-bound proteins. Unfortunately, we have no data at this point that allow us to assess the importance of this question in the present situation. Finally, the different distribution seen in various RH–di 18:1 *trans*-recombinants might originate from protein denaturation in the dark-adapted RH recombinants. This seems rather unlikely, as the aggregation seems to be lipid specific, with the extent of aggregation seemingly only a function of temperature from which the sample was frozen. We await future work to clarify some of these points.

SUMMARY AND CONCLUSIONS

We have shown that measured pair distribution functions from freeze-fracture pictures of protein-lipid recombinants resemble those calculated from models of two-dimensional fluids. Using the hard-disk model as a reference, it is possible to determine whether there are additional attractive or repulsive interactions between protein molecules reconstituted into lipid bilayers. For BR in di 12:0, di 14:0, and di 16:0 PC bilayers, EM pictures reveal no evidence for interactions between particles other than those due to the repulsive hard core. Bleached RH in di 12:0 PC and di 18:1 *trans*-PC and dark-adapted RH in di 10:0 PC show evidence of an additional repulsive interaction at longer range, which is probably electrostatic in origin and may be modeled directly as such. There appears to be, for dark-adapted RH in di 18:1 *trans*-PC, evidence for an additional attractive interaction, which we propose is lipid mediated. The most likely bilayer parameter mediating this interaction is the average lipid tilt relative to the direction of the normal to the bilayer, as tilt perturbations can be propagated over sufficiently large domains via coupled intermolecular interactions. Further theoretical and experimental studies are underway to investigate the molecular basis of this interaction.

We thank Drs. Jim Wade and George Palade for the use of the freeze-fracture apparatus and Dr. John Flory for the use of the electron microscope facilities. Dr. Jean-Paul Revel kindly provided access to digitizing and computing facilities in his laboratory, and L. Pearson is grateful to Dr. Revel and Dr. John Hopfield for numerous stimulating discussions.

This work was supported by grants GM 22432-07 (to Dr. Chan) and GM 22778 and GM 29243 (to Dr. Engelman) from the National Institute of General Medical Sciences, U. S. Public Health Service.

Received for publication 12 November 1982 and in final form 8 March 1983.

REFERENCES

Bachmann, L., and W. W. Schmitt. 1971. Improved cyrofixation applicable to freeze etching. *Proc. Natl. Acad. Sci. U.S.A.* 68:2149–2152.

Baldwin, P. A., and W. L. Hubbell. 1982. Effects of lipid environment on the decay of meta I intermediate of rhodopsin. *Biophys. J.* 37: (2, Pt. 2):85*a*. (Abstr.)

Chen, Y. S., and W. L. Hubbell. 1973. Temperature and light-dependent structural changes in rhodopsin lipid membranes. *Exp. Eye Res.* 17:517–532.

Decker, R.S., and D. S. Friend. 1974. Assembly of gap junctions during amphibian neurulation. *J. Cell Biol.* 62:32–47.

Donnell, J. T., and L. X. Finegold. 1981. Testing of aggregation measurement techniques for intra-membranous particles. *Biophys. J.* 35:783–798.

Friend, D. S., and I. Rudolf. 1974. Acrosomal disruption in sperm: freeze fracture of altered membranes. *J. Cell Biol.* 63:466–479.

Gershon, N. D., A. Demsey, and C. W. Stackpole. 1979. Analysis of local order in the spatial distribution of cell surface molecular assemblies. *Exp. Cell Res.* 122:115–126.

Huang, K. S., H. Bayley, and H. G. Khorana. 1980. Delipidation of bacteriorhodopsin and reconstitution with exogenous lipid. *Proc. Natl. Acad. Sci. U.S.A.* 77:323–237.

Lado, F. 1968. Equation of state of the hard-disc fluid from approximate integral equations. *J. Chem. Phys.* 49:3092–3096.

Marcelja, S. 1976. Lipid-mediated protein interaction in membranes. *Biochim. Biophys. Acta.* 455:1–7.

Marcelja, S., D. J. Mitchell, B. W. Ninham, and M. J. Schulley. 1977. Role of solvent structure in solution theory. *J. Chem. Soc. Faraday Trans. II.* 73:630–648.

Markovics, J., L. Glass and G. G. Maul. 1974. Pore patterns on nuclear membranes. *Exp. Cell Res.* 85:443–451.

Marsh, D., A. Watts, R. D. Pates, R. Uhl, P. F. Knowles, and M. Esmann. 1982. ESR spin-label studies of lipid-protein interactions in membranes. *Biophys. J.* 37:265–274.

Parsegian, V. A., N. Fuller and R. P. Rand. 1979. Measured work of deformation and repulsion of lecithin bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 76:2750–2754.

Pearson, R. P., S. W. Hui, and T. P. Stewart. 1979. Correlative statistical analysis and computer modeling of intramembranous particle distributions in human erythrocyte membranes. *Biochim. Biophys. Acta.* 557:265–282.

Pearson, T., and S. I. Chan. 1982. Effects of lipid-mediated interactions on protein pair distribution functions. *Biophys. J.* 37:141–142.

Perelson, A. 1978. Spatial distribution of surface immunoglobulin on B lymphocytes. *Exp. Cell Res.* 112:309–321.

Peters, R., and R. J. Cherry. 1982. Lateral and rotational diffusion of bacteriorhodopsin in lipid bilayers: Experimental test of the Saffman-Delbrück equations. *Proc. Natl. Acad. Sci. U.S.A.* 79:4317–4321.

Staehelin, L. A. 1976. Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro. *J. Cell Biol.* 71:136–158.

Stamatoff, J., R. M. Lozier, and S. Gruner. 1982. X-ray diffraction studies of light interactions with bacteriorhodopsin. *Methods Enzymol.* 88:282–286.

Tsien, R. Y., and S. B. Hladky. 1982. Ion repulsion within membranes. *Biophys. J.* 39:49–56.

Weinstein, R. S. 1976. Changes in plasma membrane structure associated with malignant transformation in human urinary bladder epithilium. *Cancer Res.* 36:2518–2524.