CELL STRUCTURE AND FUNCTION 16: 167–173 (1991)

Segregation of Modified Bacteriorhodopsin Aggregations in Reconstituted Vesicle Membrane Induced by the Change of Thermodynamical Parameters

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Key words: vesicle transport/membrane protein aggregation/thermodynamical parameters/temperature change/poly (ethylene glycol)/bacteriorhodopsin

ABSTRACT. **It was clearly shown that the change in thermodynamical parameters could cause the segregation of membrane protein aggregations in the phospholipid membrane. At first, reconstituted vesicles were prepared with a membrane protein, bacteriorhodopsin and a constituent phospholipid of biomembranes, L-α-dimyristoyl phosphatidylcholine. When the temperature of the suspension was decreased or the osmotic pressure was increased by adding poly(ethylene glycol) to this vesicle suspension at 23 degrees, the circular dichroism spectra showed a typical band indicating bacteriorhodopsin trimer formation implying their aggregation. This suggests that the aggregation of trimers proceeded by adding poly(ethylene glycol) into the vesicle suspension, just as it proceeded by decreasing the temperature. Next, vesicles were prepared with fluorescein isothiocyanate-labeled bacteriorhodopsin, photoemissive bacteriorhodopsin and L-α-dimyristoyl phosphatidylcholine. The excitation energy transfer between the two modified proteins was measured by fluorescence spectroscopy. In this case, however, when poly(ethylene glycol) was added into the suspension, the yield of the excitation energy transfer decreased. This result indicates that modified proteins aggregate separately in a segregated form in the vesicle membrane.**

It is said that each stage of the processing of glycoproteins by oligosaccharide chains takes place in each cisternae seen in the Golgi apparatus (1) and that a glycoprotein processed in one stage is transported to the next stage mediated by transporting vesicles (2), (3). However, the transporting mechanism is still unknown. In order to transport only the glycoprotein in which processing has been carried out, these glycoproteins in the membrane must aggregate to segregate from other glycoproteins that have not yet been processed. How do the aggregations of glycoproteins occur? Moreover, how do the separate aggregations of so many kinds of glycoproteins in the membrane occur?

There may be a mechanism proceeded by a specific molecule such as mannose-6-phosphate and its receptor (4–7) in the endoplasmic reticulum and the Golgi apparatus to make a certain glycoprotein aggregate. However, in this case, there would have to be so many kinds of specific molecules, each accurately corresponding with a kind of glycoprotein. Also, within each kind, there would again have to be so many specific molecules corresponding with intermediate substances which are produced in a chain of protein modifications by oligosaccharides at the cisternae of the Golgi apparatus, as well as with proteins that have not yet been modified. Thus, the possibility that the segregation of protein aggregations is controlled nonspecifically according to thermodynamical conditions where all proteins would be simultaneously influenced is examined.

Aggregation of proteins in biomembranes can be thought of as a 2-dimensional phase transition from the dispersion phase to the aggregation phase. Phase transition can be controlled by any one set of thermodynamical parameters, which satisfies the requisition that aggregations are controlled nonspecifically. Will proteins in the phospholipid bilayer membrane make the phase transition from the dispersion phase to the aggregation phase as lipids do in the phospholipid bilayer membrane? What kinds of thermodynamical parameters control the phase transition of proteins if it does occur? How is it controlled? These are important problems in investigating the mechanism of vesicle transport.

A membrane protein, bacteriorhodopsin (bR) purified from *Halobacterium halobium* forms trimers which align in 2-dimensional hexagonal lattices on the surface of the cell membrane to make what is called a purple membrane (8). bR trimer formation and its aggregation can be assumed to represent the aggregation of membrane proteins in general. If trimer formation of bR

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Abbreviations used: bR, bacteriorhodopsin; DMPC, L-α-dimyristoyl phosphatidylcholine; PEG, poly(ethylene glycol); FbR, fluorescein isothiocyanate-labeled bacteriorhodopsin; bR*, photoemissive bacteriorhodopsin.

and its aggregation can be referred to the transition from the dispersion phase to the aggregation phase, trimer formation would occur with the change of thermodynamical parameters such as temperature and pressure that are thermodynamically conjugated, as can be seen in a phase diagram. In fact, trimer formation of bR and its aggregation in reconstituted vesicles induced by decreasing temperature has been reported by Cherry *et al.* (10).

Does the increase of the surface pressure on the vesicle membrane also undertake trimer formation and its aggregation of bR? By adding poly(ethylene glycol) (PEG) into the suspension of reconstituted vesicles, the osmotic pressure of the suspension rises. The difference in osmotic pressure rises at the boundary between the bulk solution and the water layer surrounding the vesicle where there are no PEG molecules, and will work to decrease the surface area of the vesicle. By decreasing the surface area of the vesicle, the surface pressure on the vesicle membrane will increase.

Reconstituted vesicles of the consituent phospholipid of biomembrane, L-α-dimyristoyl phosphatidylcholine, and the membrane protein, bR, were prepared and PEG was added into the suspension of the reconstituted vesicles. The change of association-dispersion phase of bR by increasing the osmotic pressure which might cause an increase in the surface pressure of the vesicle membrane was observed by measuring the circular dichroism.

Another interesting problem is how the segregated aggregations of variously processed glycoproteins in the membrane occur. bR molecules can be modified by covalently adding certain kinds of molecules to simulate a model of the membrane proteins modified by oligosaccharides. Moreover, if a probe is added to bR, the change of bR's association-dispersion state could be observed by the measurements of the probe's state. The model experiment is described below.

First, bR was labeled with fluorescein isothiocyanate (FITC). Labeling bR by FITC can be thought of as simulating the modification by oligosaccharides to the membrane proteins. Next, photoemissive bR was prepared by applying the procedures of Peters *et al.* (11). In this treatment, no molecule was added to bR. Excitation energy transfer can occur between the acceptor, the FITC-labeled bR (FbR), and the donor, the photoemissive bR (bR*), because the emission wavelength of bR*, 470 nm and the absorption wavelength of FbR, 490 nm, are sufficiently close to each other (12). The changes in distance between FbR and bR* were observed from the change in the yield of excitation energy transfer.

MATERIALS AND METHODS

bR was purified in the form of purple membrane from *Halobacterium halobium* S9 by the methods of Oesterhelt *et al.* (13). Reconstituted vesicles were prepared by applying the methods of van Dijck and van Dam (14). Ten mg of L-α-dimyristoyl phosphatidylcholine (DMPC) (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in chloroform in a 100-ml Kjeldahl-type flask to form a film, evaporating the chloroform in a rotary evaporator (Shibata, RE111). Two mg of bR, suspended in 10-ml buffer solution (140 mM NaCl, 10 mM PIPES, pH 7.5) was mixed with DMPC. The suspension was sonicated for 15 min. by using a bath-type sonicator (Kokusai Electric, CLEAN MATIC), then centrifuged at 20000 g for 90 min. PEG (average M. W. 400, Wako Pure Chemical Industries) was then added to the supernatant.

The contents of protein and phospholipid were analyzed quantitatively by Miller's (15) and Allen's (16) procedures, respectively. The DMPC/bR molar ratio was 78. The size distribution of the prepared vesicles in the sample suspension was analyzed with a Malbern particle sizer 4700-PS/MW by the photon correlation method. The average diameter was around 70 nm. The vesicles were thought to be unilamellar. CD spectra measurements were performed with a JASCO spectropolarimeter J-720 using a 2 cm-path-length sample cell. The temperature of the sample cell was controlled by circulating water into the cell holder.

Fluorescein isothiocyanate isomer I (FITC) (Sigma Chemical Company, MO, U.S.A.) was mixed with bR at the molar ratio of 10 : 1 in 10 ml of buffer solution (1 M NaCl, 50 mM Na borate, pH 9.1). After the suspension was incubated at room temperature for 48 hrs., HCl was added to adjust the pH to 4.5 and the suspension was incubated at 4 degrees for several hours to terminate the dye-protein binding reaction. The suspension was then centrifuged at 50000 g at 10 degrees for 30 min. and resuspended in double-distilled water a few times.

The extinction coefficient of around 490 nm of FITC binding with proteins, e.g., insulin, is 68000 (17) and the extinction coefficient at 570 nm of native bR is 54000 (13). The molar ratio of FITC/bR calculated from each absorbance was 0.49. The treatment of bR in order to give the photoemissivity was carried out by applying the procedure used by Peters *et al.* (11). In the sample after the treatment, the peak at 570 nm characteristic of native bR disappeared and instead, a new peak appeared at 360 nm. The emission spectrum of the suspension excited with 360 nm showed a peak at around 470 nm.

One mg each of FbR and bR* was measured from the absorbance at 280 nm, the absorption of amino acids. Then, reconstituted vesicles containing FbR, bR* and DMPC were prepared by the same methods used for the preparation of bR+DMPC vesicles. In this case, the DMPC/(FbR+bR*) molar ratio was 73. The emission spectrum was measured with a Hitachi fluorescence spectrometer F-4010. The scanning bandwidth was 5 nm and the scanning speed was 120 nm/min. The temperature of the sample cell was controlled by circulating water into the cell holder.

RESULTS

Figure 1 shows the CD spectra of bR+DMPC vesicle suspension at 33, 23 and 2 degrees. As the temperature is decreased, a positive peak and a negative peak appeared at 540 nm and 600 nm, respectively. This is in accordance with the CD spectrum of purple membrane (9) and of bR trimers formed in the DMPC membrane as reported by Cherry *et al.* (10).

While increasing the amount of PEG in the vesicle suspension at a constant temperature of 23 degrees, two peaks, at 540 nm and 600 nm, appeared in the CD spectra as shown in Figure 2. These peaks were similar to what was seen at 2 degrees in Figure 1c. This implies that bR formed trimers and aggregated by adding PEG into the vesicle suspension just as it did by decreasing the temperature. Figure 3 shows the differences in ellipticity between the peaks at 540 nm and 600 nm at 23 degrees at various PEG concentrations. In Figures 2 and 3, the ellipticity was converted to per original concentration before adding PEG. A large change appeared in the region above 50% (w/w), whereas there was hardly any change from 0% (w/w) up to 50% (w/w). In fact, the difference in ellipticity at PEG concentration 80% (w/w) was larger than that at 2 degrees without PEG. From the above observations, it was confirmed that when PEG is added into the bR+DMPC vesicle suspension, bR forms trimers and aggregates in the vesicle membrane.

Figure 4 shows the emission spectrum of FbR+bR* +DMPC vesicle suspension excited at 360 nm at 23 degrees. The emission peaks of bR* and of FbR were seen at 467 nm and at 517 nm, respectively. The spectrum of FbR and bR* directly suspended in the buffer solution without the vesicles, excited at 360 nm at 23 degrees only showed the emission peak of bR* at 467 nm and no peak at 517 nm.

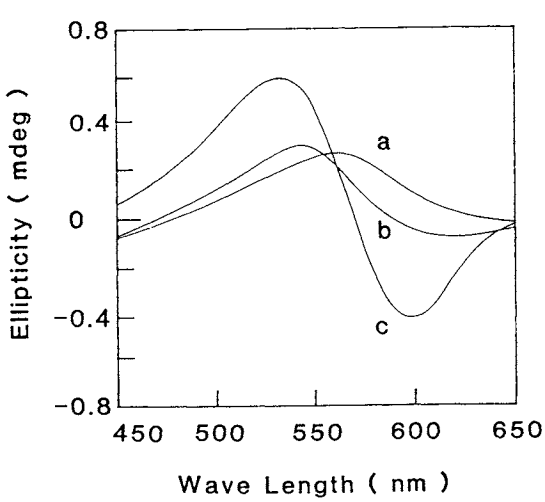

Fig. 1. CD spectrum showing the effect of temperature of bR+DMPC vesicles suspension. Curve a: at 33 degrees; curve b: at 23 degrees; curve c; at 2 degrees. With decreasing temperature, a positive peak and a negative peak appeared at 540 nm and at 600 nm, respectively.

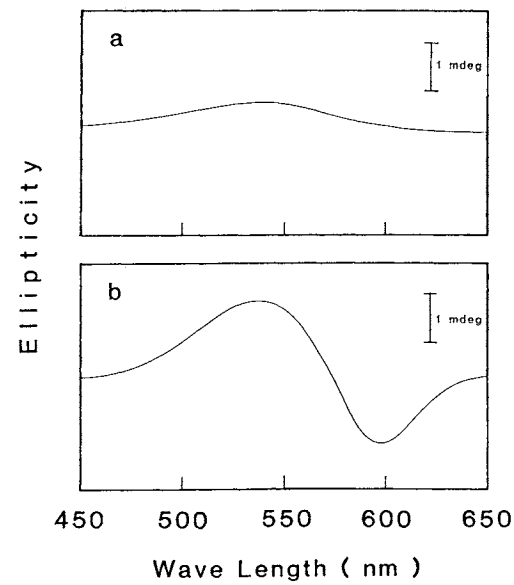

Fig. 2. CD spectra showing the effect of the addition of PEG to bR +DMPC vesicles suspension at 23 degrees. Curve a: without PEG; Curve b: at 80% (w/w) PEG. In curve b, the ellipticity is converted to per original concentration before adding PEG. Two peaks, at 540 nm and 600 nm, appeared as was seen at 2 degrees in Fig. 1.

The diameter of the prepared FbR+bR*+DMPC vesicles was around 70 nm and the molar ratio of DMPC/(FbR+bR*) was 73. Supposing the shape of the vesicles to be spherical, in the vesicle membrane the distance between FbR and bR* was calculated to be around 7 nm. In the case of the direct suspension of

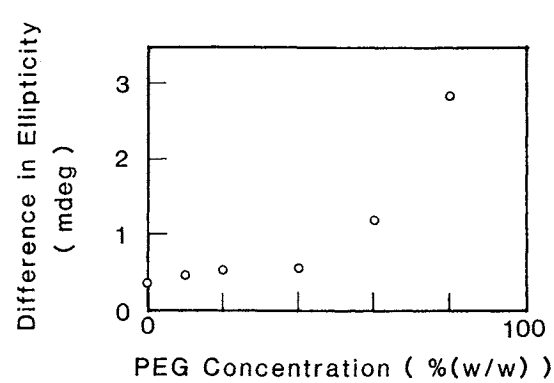

Fig. 3. The difference in ellipticity at 540 nm and at 600 nm at 23 degrees by the addition of PEG at various concentrations. Each difference is converted to the value per original concentration before adding PEG. A large change appeared above about 50% (w/w).

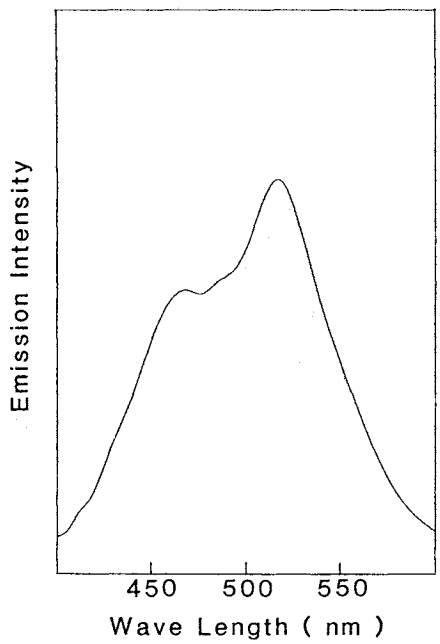

Fig. 4. Emission spectrum of the FbR+bR*+DMPC vesicles suspension. The suspension was excited at 360 nm. Emission peaks appeared at 467 nm in correspondence to the emission of bR* and at 517 nm in correspondence to the emission of FbR.

FbR and bR* not in the vesicles, with the equivalent amount of bR as in the vesicle suspension, the distance was calculated to be around 70 nm.

FbR has its excitation wavelength at 490 nm, and it cannot be excited directly by the excitation light of 360 nm. However, if FbR lies as close as several nm to bR* physically, the emission energy of bR*, corresponding to the wavelength of 467 nm, can be transferred to FbR and FbR can emit light at 517 nm (12). Consequently, these results provide evidence that the excitation energy transfer did occur between bR* acting as an energy donor, and FbR, acting as an energy acceptor in the DMPC membrane.

Figure 5 shows the effect in the emission spectrum of FbR+bR*+DMPC vesicle suspension by decreasing the temperature from 33 degrees to 4 degrees. Hardly any change occurred in the ratio of emission intensities in the range between the peaks at 467 nm and 517 nm. Figure 6 shows the emission spectra of FbR+bR*+DMPC vesicle suspension at 23 degrees with various PEG concentrations in the suspension. Each emission intensity was converted to per original concentration before adding PEG. Obvious differences were seen among the spectra by changing the PEG concentration.

The emission intensity of FbR at 517 nm was calcu-

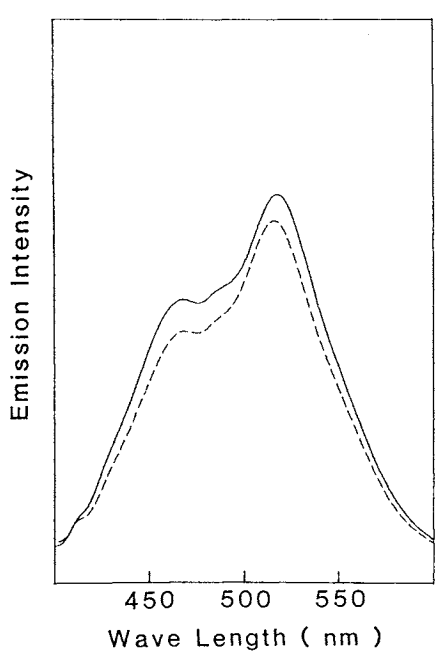

Fig. 5. The effect in the emission spectrum of the FbR+bR*+DMPC vesicle suspension by decreasing the temperature from 33 degrees to 4 degrees. The suspension was excited at 360 nm. Curve (——): at 33 degrees; curve (– – –): at 4 degrees. There was hardly any change in the ratio of emission intensity between 467 nm and 517 nm.

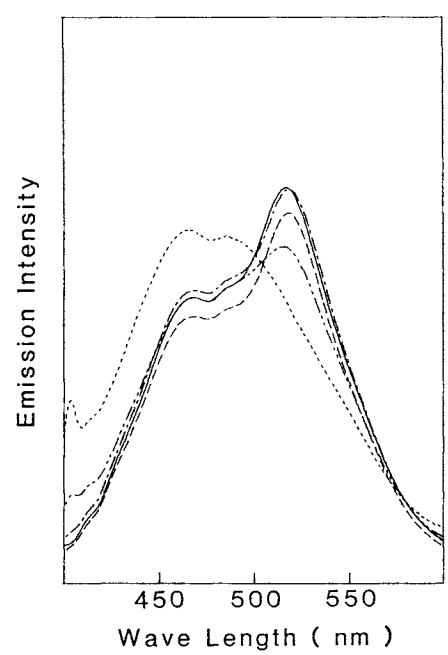

Fig. 6. The emission spectra of the FbR+bR*+DMPC vesicle suspension at various PEG concentrations at 23 degrees. Curve (——): without PEG; curve (– – –): at 20% (w/w) PEG; curve (– – –): at 40% (w/w) PEG; curve (– – –): at 60% (w/w) PEG; curve (– – –): at 80% (w/w) PEG. Each emission intensity is converted to the value per original concentration before adding PEG. With the increase in PEG concentration, the emission intensity at 517 nm disappeared, whereas the emission intensity at 467 nm increased.

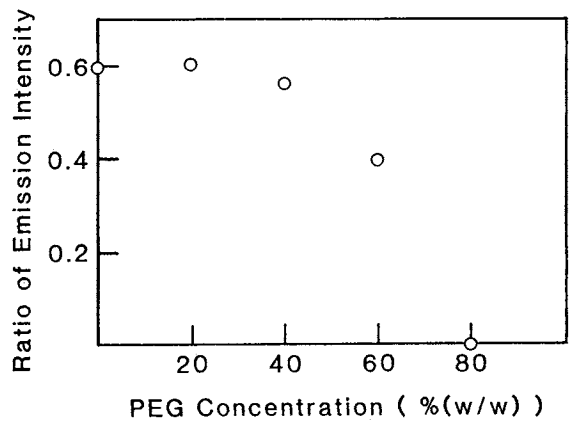

Fig. 7. The yield of excitation energy transfer from bR* to FbR in the suspension of FbR+bR*+DMPC vesicles at various PEG concentrations. The yield of excitation energy transfer decreased above the PEG concentration of 50% (w/w).

lated by substracting the emission intensity of bR* at 517 nm measured from the spectrum containing only bR*. Figure 7 shows the ratio between the emission from FbR at 517 nm and the emission from bR* at 467 nm at various PEG concentrations. The emission ratio decreased when the PEG concentration was above 50% (w/w) and the emission of FbR disappeared entirely at 80% (w/w). This result showing the decrease of the excitation energy transfer implies the increase of the distance between the donor, bR*, and the acceptor, FbR.

Eighty percent (w/w) PEG in the FbR+bR*+DMPC vesicle suspension was removed by dialyzing for 24 hrs. Figure 8 shows the emission spectrum of the FbR+bR*+DMPC vesicle suspension after PEG was removed. Each emission intensity was converted to per FbR concentration calculated from the absorbance at 570 nm. Recovery of the excitation energy transfer was observed. From this, the change in the emission spectrum by adding PEG can be thought to be a reversible process. This means that this process accompanied no breakdown of vesicles. Figure 9 shows the change of the emission spectrum of FbR+bR*+DMPC vesicle suspension at 60% (w/w) of PEG concentration by increasing the temperature from 23 degrees to 63 degrees. The peak at 517 nm, which had disappeared by the addition of PEG, recovered with the increase in temperature.

DISCUSSION

When the temperature of the suspension was decreased or PEG was added into the suspension at 23 degrees, bR formed trimers. In the liposome aggregation,

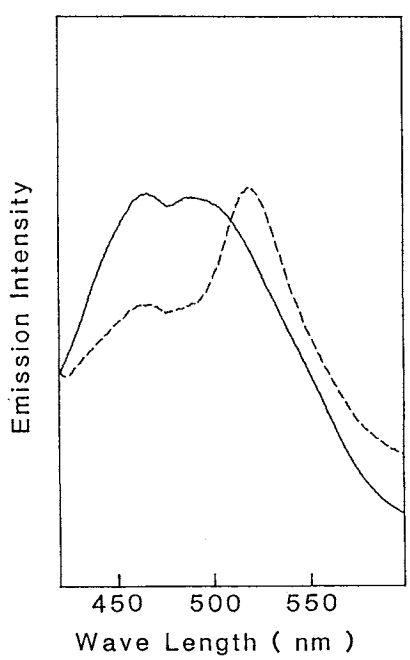

Fig. 8. The effect in the emission spectrum of the FbR+bR*+DMPC vesicle suspension by removing PEG at 80% (w/w) in the suspension by dialysis at 23 degrees. Curve (——): before dialysis; curve (– – –): after dialysis for 24 hrs. Each emission intensity was converted to per FbR concentration calculated from the absorbance at 570 nm. The emission peak at 517 nm recovered by removing PEG.

Fig. 9. The effect in the emission spectrum of the FbR+bR*+DMPC vesicle suspension with PEG of 80% (w/w) concentration by increasing temperature. Curve (——): at 23 degrees; curve (– – –): at 33 degrees; curve (– – –): at 43 degrees; curve (– – –): at 53 degrees; curve (– – –): at 63 degrees. The emission peak at 517 nm recovered by increasing the temperature.

MacDonald showed that liposome could aggregate by adding PEG only in the outer water of the liposome suspension comparted by a dialytic membrane (18) and mentioned that liposome aggregation is caused by the increase of osmotic pressure and not by the direct interaction of PEG. The difference in osmotic pressure at the boundary between the bulk solution and the water layer surrounding the vesicle where there are no PEG molecules raises the perpendicular stress to the membrane. This stress will make the membrane surface area smaller. Decreasing the membrane surface area, the surface pressure would increase.

In the case of a monolayer mixed with myristic acid and cyanine dye, the surface area is inversely proportional to the surface pressure and associators of cyanine dye are seen to appear with increasing surface pressure (19). Hence, it is reasonable that liquid-crystalline phase transition occurs in bR and DMPC bilayer membranes with increasing surface pressure at a certain temperature, as well as with decreasing temperature.

Trimer formation of FbR and bR* would occur in FbR+bR*+DMPC vesicles by adding PEG into the suspension just as it would in native bR in DMPC membranes. In FbR+bR*+DMPC vesicles, the distance between FbR and bR* dispersed randomly in the vesicle membrane is calculated to be around 7 nm. This is close enough to cause the excitation energy transfer from the donor, bR*, to the acceptor, FbR.

The yield of excitation energy transfer is proportional to the negative 6th power of the distance and to the 2nd power of the orientation factor between FbR and bR*. If the trimers contain FbR and bR* heterogeneously, the distance between FbR and bR* should be around 3 nm. The value of the orientation factor among FbR and the nearest neighboring bR* is hardly changed by the hexagonal coordination of FbR and bR* in heterogeneous aggregation. As a result, the yield of excitation energy transfer should increase. However, figure 4 shows the decrease of the yield of excitation energy transfer by adding PEG into the suspension. It is hard to believe that the extrication of FbR and bR* from vesicles is the cause of the decrease, since the energy transfer is reversible, implying that there is no breakdown of vesicles as shown in Fig. 8. Again, PEG may not be working to quench the emission of FbR because, in Fig. 9, the emission of FbR at 517 nm recovered in the presence of PEG at 60% (w/w). Hence, the remaining possibility is that each trimer is composed of homogeneous molecules, containing only FbR or bR*.

Supposing the trimers are composed homogeneously with either one of FbR or bR* and the other dispersed in the vesicle membrane, the average distance between FbR and bR* in the vesicle membrane would increase to several multiples of 7 nm, the distance in the case of random dispersion. Also, in the case where FbR and bR* form homogeneous trimers separately, the average distance between FbR and bR* would increase to about 10 times the distance in random dispersion. The distances in both of the above cases are large enough for the excitation energy transfer between FbR and bR* to vanish. Thus, from the above observations, it can be said that at least either FbR or bR* aggregates homogeneously, excluding the other.

Tilcock and Fischer had reported that the main phase transition temperature of dipalmitoyl phosphatidylcholine bilayer membrane rises in the PEG concentration above 50% (w/w) (20), and mentioned that almost no free water is left at this concentration because PEG hydrates approximately the same mass of water as itself. In our case, in the bR+DMPC vesicles and the FbR+bR*+DMPC vesicles, the effect of PEG also appeared above about 50% (w/w). This suggests that the PEG concentration above 50% (w/w) might change the condition of the lipid membrane to decrease the surface area and form bR trimers or segregated aggregation of FbR and/or bR*.

In the case of FbR+bR*+DMPC vesicles, the segregated aggregation of FbR and/or bR* did not seem to occur by the change of the temperature from 33 degrees to 4 degrees, as shown in Fig. 6, although the bR trimer formation and its aggregation in bR+DMPC vesicles occurred by the change of the temperature from 30 degrees to 2 degrees as was shown in Fig. 1. The bR trimer formation and its aggregation in DMPC membranes occurs in the range of within 10 degrees to around 23 degrees, which is where the liquid-crystalline transition temperature of DMPC lies (21), (22). Thus, in the case of native bR in DMPC membranes, increase of temperature by about 10 degrees is enough to make the bR trimers disperse. In contrast, the dispersion of FbR and/or bR* in vesicles at 60% (w/w) PEG concentration was not complete even with the increase of temperature by about 40 degrees. However, the segregated aggregation of FbR and/or bR* did occur by the addition of PEG. Thus, the segregated aggregation of FbR and/or bR* should occur at a temperature below 4 degrees, *i.e.*, by a temperature change of more than 40 degrees.

Comparing the temperature differences, 40 degrees and 10 degrees, required for the transition of FbR and/or bR* and native bR, respectively, the energy required for the transition of FbR and/or bR* is thought to be larger than the energy required for the transition of native bR. This is probably due to the change of the potential energy profile of intermolecular forces by adding FITC or by denaturation. It is reasonable to assume that FbR and bR* aggregate segregatively in DMPC membranes if these bR molecules have different potential energy profiles by the modification of the molecules.

It can be said that membrane proteins could be ag-

gregated; moreover, the segregation of protein aggregations according to the kinds of proteins could be induced by the change of thermodynamical parameters, for example, temperature or pressure which were confirmed in the discussion as described. This reveals the possibility for the study of the mechanism of vesicle transport.

Acknowledgments. We would like to thank Mr. A. Takakuwa (Japan Spectroscopic Co., Ltd.) for his support in measuring circular dichroism spectra by using the J-720 spectropolarimeter and Mr. T. Fujii and Prof. T. Yano (Department of Agricultural Chemistry, University of Tokyo) for their support in measuring the size of vesicles by using a Malbern particle sizer 4700-PS/MW. This work was partially supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

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(Received for publication, December 20, 1990
and in revised form, March 13, 1991)