Spatial Organization of Bacteriorhodopsin in Model Membranes

LIGHT-INDUCED MOBILITY CHANGES*

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Bacteriorhodopsin is a proton-transporting membrane protein in Halophilic archaeca, and it is considered a prototype of membrane transporters and a model for G-protein-coupled receptors. Oligomerization of the protein has been reported, but it is unknown whether this feature is correlated with, for instance, light activation. Here, we have addressed this issue by reconstituting bacteriorhodopsin into giant unilamellar vesicles. The dynamics of the fully active protein was investigated using fluorescence correlation spectroscopy and freeze fracture electron microscopy. At low protein-to-lipid ratios (<1:10 w/w), a decrease in mobility was observed upon protein photoactivation. This process occurred on a second time scale and was fully reversible, i.e. when the dark-adapted state was reestablished the lateral diffusion rate of the protein was returned to that prior to activation. A similar decrease in lateral mobility as observed upon photoactivation was obtained when bacteriorhodopsin was reconstituted at high protein-to-lipid ratios (>1:10 w/w). We interpret the shifts in mobility during light adaptation as being caused by transient photoinduced oligomerization of bacteriorhodopsin. These observations are fully supported by freeze-fracture electron microscopy, and the size of the clusters during photoactivation was estimated to consist of two or three trimers.

Halophilic archaea are salt-loving microorganisms that use bright sunlight as a source of energy (1). For this purpose, they host in their cell membrane bacteriorhodopsin (2, 3) a 26-kDa transmembrane protein that converts light into an electrochemical proton gradient. Bacteriorhodopsin (BR) is considered to be a prototype for a membrane transporter (4) and also serves as a structure model for G-protein-coupled receptors. BR and G-protein-coupled receptors share in common a seven-helix topology, and both are thought to operate by a similar switching mechanism (5–7). The overall mechanism of proton pumping is well understood, and three-dimensional structures at high resolution are available for the ground state (8) as well as for several intermediate states of the photocycle (9–12). Thus, the structural changes that push a single proton through the seven-helix bundle from the cell interior to the extracellular milieu (13, 14) have been described with atomic detail. An intriguing question that remains to be resolved is whether the spatial organization of BR, which forms two-dimensional hexagonal crystals of trimers in the native membrane (2), plays a functional role in proton transport. Such a change in organization could be instrumental, for example, in promoting or regulating the proton-pumping rate or proton transport, and several hypotheses have been proposed in this regard, but no consensus has been reached yet (15–17). Although the hexagonal packing does not appear to be required for the function of a protein (15), the question as to whether aggregation into a trimer or larger oligomeric structure underlies a functional property has yet to be addressed.

The aggregation state of BR has been investigated by several techniques. In the CD spectrum, the purple membrane exhibits a characteristic excitonic signal caused by interactions between the protein chromophores in the closely packed hexagonal lattice (18). A similar signal is detectable in BR, reconstituted into liposomes, and analyzed below the phase transition temperature (Tc) of the phospholipids used (15, 19). However, above Tc, the excitonic effects disappear, and only at high protein-to-lipid ratios (>1:10 w/w) is a considerable amount of exciton coupling observed (19). The disappearance of the exciton component in the CD spectrum has been interpreted as dissociation of the aggregate, yielding a monomeric dispersion of BR in the plane of the membrane (15). Analogous conclusions were drawn from measurements of anisotropy decay of transient absorption changes (19).

Based upon a freeze fracture electron microscopy study (20), the monomeric state of BR in proteoliposomes was questioned. In fact, such a state was only observed at very low protein-to-lipid ratios (<1:40 w/w), whereas self-association of BR occurred at protein-to-lipid ratios at which the excitonic feature is not yet detectable in the CD spectrum. Evidently, the latter feature is of insufficient sensitivity to detect the early presence of smaller oligomers in BR liposomes. In the EM studies, aggregation of BR in the dark/light-adapted state was not investigated (20).

Here, we report for the first time on the lateral mobility and
the state of molecular association of BR, reconstituted into giant unilamellar vesicles (GUV), i.e. model membranes that display cell-like dimensions. We show by fluorescence correlation spectroscopy (FCS) (21, 22) that at low protein-to-lipid ratios (<1:10 w/w), a mobility change is associated with BR photoactivation. These findings on transient BR aggregation during light adaptation are confirmed by electron microscopy.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**
- t-Octyl-Dioleyl-phosphatidylcholine (DOPC), t-octyl-phosphatidylethanolamine (DOPE), and t-dipalmitoyl-phosphatidyl-
- diolethanolamine were purchased from Avanti Polar Lipids. N-Succin-
- imidyl 3-(2-pyridyldithio)-propionate-derivatized L-

**Activity of Bacteriorhodopsin in GUV—**Changes in the pH of the lumen of the GUV caused by the proton pumping of the reconstituted bacteriorhodopsin were measured as changes in the fluorescence intensity of the membrane-impermeable, pH-sensitive probe pyranine (25). Bacteriorhodopsin was reconstituted into WAE-coupled LUV suspended in 20 mM Pipes, pH 7.2, and containing 1% of Texas Red DHPE. The LUV were fused to GUV prepared in 20 mM Pipes, pH 7.2, 200 μM pyranine, and 100 mM valinomycin. Fusion was checked by monitoring lipid mixing under the microscope, reflected by diffusion of the Texas Red DHPE (λem = 582 nm and λexc = 601 nm) into the membrane of the GUV. After 30 min of illumination with a xenon lamp, the GUV caused by the proton pumping activity of bacteriorhodopsin was observed with the microscope as a decrease in the fluorescence intensity of pyranine entrapped in the GUV. The same activity assay was reproduced in LUV, and under the same experimental conditions as for GUV, an initial proton pumping rate of 30 nequiv of H+ (total proton uptake of 600 nequiv of H+) was observed at protein-to-lipid ratios of 1:40 (w/w), and an initial proton pumping rate of 390 nequiv of H+ (total proton uptake of 610 nequiv of H+) was observed at protein-to-lipid ratios of 1:3 (w/w).

**Fluorescence Correlation Spectroscopy—**

The experimental set-up consisted of a continuous wave argon ion laser operating at 488 nm. The laser beam was directed via a dichroic mirror and a 63× numerical aperture 1.2 water immersion objective (Zeiss C-Apochromat) to the sample (3.8 × 300 Wcm⁻²), and the fluorescence was collected by the same objective and imaged onto a 30-μm pinhole located in front of an avalanche photodiode (EG&G, Canada). An OG515 filter placed in the detection arm filtered out the excitation light, back-scattered from the sample. The signal was sent to a PCI-6602, 80 MHz counter card (National Instruments) and then to a computer where the algorithm for the autocorrelation curve was applied. To measure the lateral mobility of lipid and protein in the membrane, a two-dimensional diffusion model was used (31). For noninteracting particles undergoing two-dimensional translational Brownian motion with different mobilities and/or different emission rates, the general expression for the autocorrelation function is as follows (22, 31).

\[
A_C(r,t) = \sum_{n} \frac{\kappa_n^2}{\lambda_n^2} \left[ \left( \frac{r}{\lambda_n} \right)^{2n} \frac{\lambda_n^2}{\lambda_n^2} \right] \exp \left( -2 \frac{r^2}{\lambda_n^2} \right) \]  

\[ \times \sum_{a} \frac{\kappa_a^2}{\lambda_a^2} \left( 1 + \frac{r^2}{\lambda_a^2} \right) \exp \left( -2 \frac{r^2}{\lambda_a^2} \right) \]  

\[ \text{Eq. (1)} \]

where \( \kappa_n \) is the particle emission rate, \( \langle C \rangle \) is the average local concentration, \( V \) is the effective probe volume, and \( \tau_{\text{rel}} \) is the time decay constant of the correlation curve, which is related to the diffusion coefficient as follows.

\[
\tau_{\text{rel}} = r^2 / 6D_n \]

\[ \text{Eq. (2)} \]

where \( r \) is the half-axis of the probe volume.

In case of deviations from normal motion (22), so-called anomalous diffusion, the expression \( \tau_{\text{rel}} \) is replaced by \( \tau_e / \tau_{\text{rel}} \); \( \tau_e \) is the anomalous diffusion exponent. No conventional diffusion constants can be defined in this case, and \( \tau_{\text{rel}} \) is related to a transport coefficient of fractional time dimension \( \Gamma \), as follows.

\[
\tau_{\text{rel}} = r^2 / 6\Gamma \]

\[ \text{Eq. (3)} \]

The lateral (\( r \)) and axial (\( z \)) radii of the effective volume element are \(~0.3\) and \(~0.8\) μm, respectively, as determined by imaging PS-speck 175-nm yellow-green beads (Molecular Probes) in water.

**Freeze Fracture Electron Microscopy—** BR was reconstituted at various protein-to-lipid ratios (ratio of 10 to 3:1) into LUV (size, 400 nm) with a lipid composition analogous to that of GUV after fusion (74% DOPC, 17% DOPE, 8% SAINT-2, and 1% PE-DOPC). Freeze fracture replicas were prepared from pelleted proteoliposomes that were frozen into liquid nitrogen and then thawed at room temperature to increase the vesicle size. Next, the
proteoliposomes (10 mM lipid suspension) were washed with 20% glycerol in 25 mM phosphate, pH 6.9, and placed onto gold holders. Part of the sample was kept in the dark, and part was exposed to actinic illumination for 2 min before plunging them into liquid propane (−189.7 °C) kept cool by liquid nitrogen. The frozen samples were fractured with a diamond knife (Balzers BA301) at 10⁻⁷ torr. WT/αs was evaporated and deposited at 45° elevation immediately after fracturing, and all replicas were then backed with carbon from 90°. The replicas were washed in diluted chromic acid for 3 h and picked up on 400-mesh uncoated grids for examination with the electron microscope (Philips CM10). The images enlarged at a magnification of 200,000× were digitized, and the particle diameters were measured edge-to-edge in a direction perpendicular to the shadow direction, using software from ScionImage. The precision of the measurements was estimated to be about 0.1 nm, which corresponds to an error in particle size measurement of about ± 0.5 nm.

RESULTS

The Lateral Mobility of Bacteriorhodopsin Changes upon Photoactivation—The mobility of bacteriorhodopsin in the plane of the membrane gives information on its spatial organization and aggregation state. To determine its dynamics without potential interference of adjacent proteins or an intracellular network, we have reconstituted bacteriorhodopsin into model membranes, i.e. GUV, which are most suited for single-molecule optical microscopy. A scheme of the reconstitution procedure used (25) is depicted in Fig. 1A. The protein is first inserted into 100-nm LUV, to which a fusogenic peptide (black) is coupled. The GUV contain a cationic lipid, SAINT-2, which acts as docking site for the peptide-coupled BR-containing LUV. B, BR translocation into GUV is monitored by protein-lipid mixing, as assayed by fluorescence microscopy. Here, Alexa Fluor 488-labeled BR (50 μg/ml) was reconstituted into LUV. Note the presence of the continuous ring of fluorescence, associated with GUV after fusion. Bar, 20 μm.

Although we were interested in probing protein dynamics in the plane of membranes, a three-dimensional confinement of the excitation beam in the sample was needed to produce a tight focus onto the focal plane and to maximize the signal-to-noise ratio. A scheme of the confocal microscopy set-up used in the present study is shown in Fig. 2.

At low protein-to-lipid ratios (1:300 up to 1:20 w/w), the translational mobility of bacteriorhodopsin in the dark-adapted state resulted in the autocorrelation decay shown in Fig. 3 (black circles). The excellent signal-to-noise ratio of the autocorrelation function allowed us to determine in detail the appropriate diffusion model. In a first approach, we fitted the experimental decay curve by assuming a single diffusing species undergoing Brownian motion. As shown in Fig. 3 (dashed curve), this fitting model failed. However, by using an anomalous diffusion model, the fitting perfectly matched. The experimental data and the obtained anomalous diffusion exponent, α = 0.8, and the transport coefficient, Γ = 0.8 μm²/μs, were highly reproducible (Fig. 3, solid curve). Using a two-component Brownian diffusion model, an equally excellent fitting was obtained where the two terms correspond to diffusion constants of 1.0 μm²/μs (70%) and 0.08 μm²/μs (30%) (not shown).

Intriguingly, at the same protein-to-lipid ratio, a striking shift to a lower mobility was observed upon protein photoactivation (Fig. 3, open circles). Also in this case, an excellent fitting was obtained with the anomalous diffusion model (α = 0.8 and Γ = 0.2 μm²/μs) (Fig. 3, dashed and dotted curve) or the two-component Brownian diffusion model (D₁ = 0.2 μm²/μs for 70% and D₂ = 0.08 μm²/μs for 30%) (not shown). The effect of photoactivation on reducing protein mobility was maximal when exposing the protein to the light-adapted state after 2 up to 4 min of xenon illumination. Thus, upon photoactivation the diffusion rate constant of the major fraction (70%) decreased 5-fold, whereas the (very slow) mobility of the minor fraction (30%) was not affected. Most interestingly, however, the transition from a high mobility in the dark state to a low mobility in the light-adapted state was fully reversible. Thus, when the xenon lamp was switched off again, a relaxation to the dark-adapted state with high mobility was observed on approximately the same time scale of the reverse process, and a decay curve was obtained that was indistinguishable from the one recorded prior to light activation (Fig. 3).

The data thus suggest that photoactivation may have caused a clustering of BR molecules, leading to a decrease in the rate of lateral diffusion, whereas a return of the sample to the dark caused their dissociation, thereby restoring the situation to
that obtained prior to light activation. To further investigate this possibility, the same series of experiments were repeated for samples with high protein-to-lipid ratios (1:10 up to 1:3 w/w). As shown in Fig. 4, in this case no significant difference in mobility was observed between the dark- and light-adapted state. The diffusional mobility, calculated by using the fitting model for anomalous diffusion or a two-component Brownian diffusion, revealed that the lateral diffusion rates at these conditions of an enhanced local protein concentration were virtually identical to those obtained at low protein concentrations in the light-adapted regime \( (\alpha = 0.8, \Gamma = 0.2 \mu m^2/s) \).

Finally, the observed changes in diffusion rates are results of a dynamical process on a second time scale. When recording time-lapsed autocorrelation traces of BR dynamics upon photoactivation, at least one intermediate state could be detected, corresponding to diffusion constants \( D_1 = 0.7 \mu m^2/s \) for 77% and \( D_2 = 0.08 \mu m^2/s \) for 23%. As can be inferred from Fig. 5, the half-time for the kinetics of the shift of the diffusion constant (first component \( D_1 \)) upon light activation is ~65 s.

Translational Mobility Changes Are Associated with BR Photoactivation—Although the reversibility of the observed lateral mobility switch, following a light/dark cycle, would support the reliability of the data, several control experiments were carried out to further exclude potential artifacts. First of all, a common source of errors in fluorescence correlation spectroscopy is the photobleaching of the dye molecules. This would artificially decrease the dwell time of the particles in the probe volume and, therefore, result in an apparent increase in mobility (33). To eliminate this possibility, we measured the mobility of BR at different excitation powers, determined the threshold below which the mobility was constant, and set the laser power for our measurements below that threshold (data not shown; see “Experimental Procedures”). Furthermore, the lateral mobility of two fluorescent lipid analogues, fluorescein-labeled DHPE and N-Rh-PE, which were incorporated separately into GUV at 0.1 mol % each, was measured by FCS under the same conditions as for BR, i.e. during or prior/after xenon illumination. The autocorrelation trace was fitted with a one-component Brownian diffusion model, and the calculated mobilities were 20 \( \mu m^2/s \) in either case. Finally, the effect of pH on the spectral properties of Alexa Fluor was carefully investigated; the fluorescence quantum yield and the absorbance maximum were constant between pH 3 and 10 and shifted dramatically only at pH > 10. Therefore, we conclude that the light/dark cycle-dependent mobility changes for bacteriorhodopsin reflect a
genuine shift in mobility, presumably because of a transient clustering of the protein. The initial rate of proton pumping of bacteriorhodopsin was the same at protein-to-lipid ratios of 1:40 (w/w) and 1:3 (w/w), that is, under conditions where the apparent aggregation state in the dark is different for these two protein-to-lipid ratios. Although upon illumination the protein at protein-to-lipid ratios of 1:40 (w/w) starts to aggregate, the kinetics of this process is relatively slow; proton pumping rates could already be estimated from the proton uptake after 20 s, that is, before the majority of protein at protein-to-lipid ratios of 1:40 had aggregated. We thus conclude that under our experimental conditions, the specific proton pump activity of bacteriorhodopsin is not influenced by the aggregation state.

Light-activated Clustering of BR as Visualized by Electron Microscopy—In principle, membrane proteins in artificial membranes can be readily visualized by EM as intramembranous particles in a relatively smooth lipid surface area. To obtain further support for a reversible switch in BR clustering, the spatial distribution of BR in artificial membranes was examined under the same conditions as above, prior to and during photoactivation. In Fig. 6, typical images are shown, obtained for BR-reconstituted vesicles at low protein-to-lipid ratio (1:300 w/w). Strikingly, in these samples, there was a clear difference in the size of intramembrane particles when comparing the spatial distribution of BR in the dark-adapted (Fig. 6, A and C) and light-adapted states (Fig. 6, B and D). The results of particle size analyses for such preparations are summarized in a histogram in Fig. 8A. The various particle sizes are dispersed according to a multimodal distribution, reflecting the presence of clusters of BR of different sizes. Whereas in the dark-adapted state the weighted average of the size distribution was $6.0 \pm 0.5$ nm, the value for the light-adapted state was $10.5 \pm 0.5$ nm. If it is assumed that, in the dark-adapted state, BR is dispersed as monomers or relatively small oligomers, e.g. trimers (2, 20), this spatial organization rearranges upon photoactivation into a more complicated pattern of clusters of molecules of different sizes. Moreover, as also shown in Fig. 7, the size distribution of the preparations at high protein-to-lipid ratio (1:3 w/w) was mainly determined by the protein concentration, that is, the ratio between low and high molecular weight complexes decreased with increasing protein concentration (see also Ref. 20). Particle size analyses, carried out for these conditions, are shown in Fig. 8B. Evidently, in this case the size distributions for the dark- and the light-adapted states are very similar and are centered around $10.0 \pm 0.5$ nm. Accordingly, it is evident that upon protein photoactivation, factors other than protein density appear to control the intrinsic affinity between monomers/trimers.

Taken together, the freeze fracture electron microscopy data are entirely consistent with the data obtained in the FCS experiments, implying that, upon photoactivation, BR becomes clustered, whereas a dissociation to their native state (prior to activation, in the dark) is seen when the light is switched off. Importantly, BR reconstituted into GUV is functionally active,
as was shown by pH-sensitive decreases of fluorescence of pyranine, entrapped in the GUV (data not shown), as described in detail previously (25). The presented data thus indicate that the clustering of bacteriorhodopsin reflects a functional property of the protein.

**DISCUSSION**

In this work, we have demonstrated that upon photoactivation of bacteriorhodopsin, the distribution of the protein is reorganized from monomers/trimers into larger oligomers, a process that is entirely reversible upon interruption of the light-activated state. Because the protein has been functionally reconstituted, our data imply that upon light-activated conditions, the protein acquires a multimeric organization and likely consists of two or three trimers (Fig. 8; see below).

In the native membrane, BR forms a hexagonal lattice of trimers (2). Reconstitution with synthetic lipids at protein-to-lipid ratios lower than in the native state causes the loss of the reticular order, but the protein retains its proton pumping activity (15). A long-standing issue is whether the lattice or an oligomeric organization in the membrane plays a role in the regulation of the proton pumping. Here, we show that the aggregation from monomeric/trimeric to a larger oligomeric form has no effect on the specific proton pumping activity of BR.

There is increasing evidence that transport and signal transduction proteins, in general, form functionally relevant oligomeric complexes in membranes (34). In case of G-protein-coupled receptors, of which BR is considered a model (35), the functional significance and the dynamics of their known oligomerization remain to be elucidated (36, 37). Hence, the present observations concerning BR dynamics may bear potential relevance to functional mechanisms of structurally similar proteins, such as G-protein-coupled receptors.

Here, we have shown for the first time that the spatial organization of BR correlates with its state of photoactivation. At fairly low protein concentrations, the protein in the dark-adapted state is dispersed as well defined small particles, whereas in the light-adapted state the protein rearranges into much larger clusters. At higher protein-to-lipid ratios, the spatial organization is mainly determined by the local density of the protein. No larger clusters could be observed upon photoactivation, thus suggesting that as a result of factors that remain to be determined, a cluster size limit exists. Although the data strongly support the view of photoactivation-induced clustering of BR as the cause of changes in its lateral mobility, it is possible that illumination-induced conformational changes could contribute as well. However, unrealistically large conformational changes would then be required, following photoactivation. Arguments based on a hydrodynamics theory (38) indicate that differences in mobility, as reported here (light versus dark), should elongate the molecule at least 9–14 times along the direction parallel to the plane of the membrane. X-ray crystallography studies of intermediate states of BR during the photocycle exclude this possibility (10, 13).

In the past, several studies have been carried out to determine the oligomerization state of BR in artificial liposomes (15–17), and the discrepancies found, for instance between circular dichroism and freeze fracture electron microscopy, are most likely due to the relatively poor sensitivity of the techniques (20, 39, 40). In particular, there are good reasons to believe that the excitonic feature in the CD spectrum appears only upon extended exciton coupling in large BR oligomers or in the crystal, but the (in)sensitivity of the method precludes to unequivocally probe the state of aggregation. In this respect, FCS is the preferred technique to study in detail the diffusional dynamics of bacteriorhodopsin and is sensitive enough to monitor protein mobility at very low protein-to-lipid ratios and to detect association phenomena at conditions of a very limited degree of oligomerization.

The FCS data can be properly fitted by using an anomalous diffusion model or a two-component Brownian diffusion model. It is possible that the deviation from normal diffusion is the result of different, coexisting diffusion coefficients or, alternatively, may result from the occurrence of protein-protein and lipid-protein interactions that give rise to correlated motions and anomalous subdiffusion. In the case of the two-component Brownian model, the slowest component (fraction of 30%) essentially reflects the presence of an immobile fraction. Note that this fraction does not reflect the presence of docked, nonfused, LUV on the GUV surface, because the FCS curves were recorded from regions of GUV with a smooth fluorescence signal. Accordingly, this fraction might indicate the presence of immobile BR clusters in the GUV membrane.

Intramembrane particle size analysis conducted on freeze fracture electron micrographs revealed a broad and multimodal size distribution. The weighted average for a typical size distribution of a sample at a low protein-to-lipid ratio and in the dark-adapted state is about 6.0 ± 0.5 or 4.3 ± 0.5 nm, when corrected for the thickness of deposited metal film (1.7 nm).
Because the cross-sectional area of a single BR obtained by x-ray is 2.5 × 3.2 nm², this size distribution would match that of a population of monomers and trimers. At the same protein-to-lipid ratios, light-adapted BR particles have a weighted average size of 10.5 ± 0.5 nm, corrected to 8.8 ± 0.5 nm, which would correspond to an average cluster of six to nine subunits. Eskandari et al. (41) have found a linear correlation between the protein cross-sectional areas derived from two-dimensional crystals and the number of membrane-spanning α-helices, each helix occupying on average 1.40 ± 0.03 nm². Particles with a size distribution around a corrected radius of 4.3 ± 0.5 nm would thus contain 10.5 helices on average and therefore, correspond to a combination of monomers (seven transmembrane α-helices) and trimers, whereas a corrected average radius of 8.8 ± 0.5 nm corresponds to 44 helices on average. Given the multimodal complexity of the size distributions obtained, especially in the light-adapted state, these are only rough indications of an average size but are consistent with the hypothesis of BR assemblies of two or three trimers in the light-adapted state.

Next to a qualitative agreement between FCS and EM, we also considered a quantitative comparison of the data. From EM, it can be determined that the ratio between the average particle radii in the light-adapted (8.8 ± 0.5 nm) and dark-adapted states (4.3 ± 0.5 nm) is ~2. By FCS, particle radii are not directly measured, but rather diffusion constants and the ratio between the diffusion constants of BR in the dark-adapted (0.8 μm²/s) and in the light-adapted states (0.2 μm²/s), respectively, is ~4. If we assume a Brownian diffusion model of spherical particles diffusing in a continuous medium, the Einstein relation holds (42).

$$D = \frac{kT}{6\pi\eta R}$$

where $k$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the medium, and $R$ is the sphere radius. In this frame, the ratio between diffusion constants becomes $D_1/D_2 \sim \eta_1/\eta_2 \cdot R_1/R_2 \sim 4$. In a case where the viscosities are the same, $R_1/R_2 \sim 4$, which represents an offset with respect to the ratio obtained by freeze fracture electron microscopy (~2). However, the fitting of the FCS data, according to the anomalous diffusion model, indicates that the protein dynamics do not correspond to a simple Brownian diffusion of spherical particles in a continuous medium. The situation is more complicated and appears to involve a more complex hydrodynamical model in which the lipids may play an important role, which could cause the offset between the two techniques. As indicated in several previous studies (3, 43, 44), protein-protein interactions for bacteriorhodopsin are lipid-mediated. A role of the lipids in the aggregation phenomenon should be considered not only in terms of specific lipid-protein interactions (in the experiments reported here bacteriorhodopsin retains part of the tightly bound native lipids) but also in terms of collective lipid properties, such as viscosity and domain formation. This might explain the discrepancies often observed among preparations with different lipid compositions.

Finally, it is of relevance to briefly discuss the structural basis of BR clustering upon photocactivity. Clustering occurs already at a very low protein-to-lipid ratio and, given that the GUV is essentially composed of DOPC, is very likely driven by an intrinsic affinity of the protein for itself, rather than being caused by protein segregation from the bulk lipids into a lipid microdomain. This behavior might reflect the tendency of the protein to increase the self-association during proton pumping to increase the stability of the protein. We do not have indications that BR in the aggregated state (two or three trimers) has a higher specific activity than the trimer. The structural mechanism for the increase in protein-protein affinity and formation of transient large clusters can be manifold. X-ray crystallography, cryo-EM, and EPR studies have clearly shown the occurrence of significant conformational changes of BR during the photocycle, such as bending of certain helices and cooperative movements of the external soluble loops (10, 12, 13, 45). These structural changes might act simultaneously together with changes in specific protein-lipid interactions, but also changes in the solvation shell around the soluble loops caused by local pH changes may lead to a global increase of the protein self-affinity.

In conclusion, oligomerization of bacteriorhodopsin, spontaneously occurring upon photoactivation, might increase the efficiency or facilitate the regulation of the proton transport. The present work merits further investigation on this signal-triggered spatial reorganization, which may be a feature common to many other cell membrane receptors involved in signaling and transport pathways.

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