Selinger, B. K., & Harris, C. M. (1983) in *Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology* (Cundall, R. B., & Dale, R. E., Eds.) p 729, Plenum, New York.
Slaunwhite, W. R., Jr., Engel, L. L., Olmsted, P. C., & Carter, P. (1951) *J. Biol. Chem. 191*, 627–631.
Spencer, R. D., & Weber, G. (1970) *J. Chem. Phys. 52*, 1654–1663.
Weinreb, A., & Werner, A. (1974) *Photochem. Photobiol. 20*, 313–321.
Weinreb, A., Werner, A., & Kalman, Z. H. (1977) *Photochem. Photobiol. 26*, 567–572.
Weller, A. (1955) *Z. Phys. Chem. (Munich) 3*, 238–254.
Weller, A. (1958a) *Z. Phys. Chem. (Munich) 15*, 438–453.
Weller, A. (1958b) *Z. Phys. Chem. (Munich) 17*, 224–245.
Weller, A. (1961) *Prog. React. Kinet. 1*, 189–214.

Spontaneous Transmembrane Insertion of Membrane Proteins into Lipid Vesicles Facilitated by Short-Chain Lecithins†

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ABSTRACT: Functional reconstitution of the membrane protein bacteriorhodopsin into lipid vesicles is achieved by mixing aqueous suspensions of long-chain lecithins and purple membrane with the short-chain lecithin diheptanoylphosphatidylcholine (20 mol % of total lipid). The membrane protein is transmembranously inserted in the lipid bilayer of the vesicle and highly active as a light-energized proton pump. This rapid, easy, and gentle procedure might allow functional reconstitution of other membrane systems and isolated membrane proteins as well.

Reconstituted protein–lipid vesicle systems are one of the most successful tools for the investigation of the structure and function of membrane proteins. Either the properties of the membrane protein of interest can be studied directly in these cell-like vesicles by means of various biochemical and biophysical techniques or the reconstituted vesicles are used as starting material for the formation of planar lipid bilayers containing transmembrane proteins suitable for electrical methods. A variety of approaches have been previously developed for functional reconstitution of membrane proteins into lipid vesicles [for recent reviews, see Racker (1979), Montal et al. (1981), and Eytan (1982)]. These approaches usually comprise at least one step which may be harmful for the membrane protein to various degrees, e.g., prolonged sonication, solubilization by and subsequent removal of detergents, and freezing and thawing. A reconstitution procedure avoiding any harmful treatment would obviously be an advantage, especially if it is easy and fast in addition. Recently, Gabriel and Roberts (1984) described a technique for spontaneously forming unilamellar lipid vesicles by mixing aqueous suspensions of long-chain lecithins (fatty acid chain lengths of 14 carbons or longer) with small amounts of micellar synthetic short-chain lecithins (chain lengths of 6–8 carbons). In the present report, it is shown that this technique is also an easy, rapid, and gentle way for successful membrane protein reconstitution. Bacteriorhodopsin (BR)[1] in the purple membrane (PM) of *Halobacterium halobium* is used as a model system in combination with different long-chain lecithins to demonstrate this fact. PM can be isolated without the use of detergents in pure form and contains as the sole protein the light-driven H^+ pump BR, which is only associated with seven native lipids. BR represents one of the best characterized transmembrane proteins, and its structural and functional properties can be easily monitored during reconstitution. In addition, several reconstitution procedures have been applied to BR (all of which require harsh treatments such as detergents, prolonged sonication, freeze–thawing, etc.) and therefore can be compared with the one under investigation (Hwang & Stoeckenius, 1977; Racker, 1979; Racker et al., 1979; Casadio & Stoeckenius, 1980; van Dijck et al., 1981; Heyn & Dencher, 1982; Rigaud et al., 1983). Upon mixing all constituents, i.e., PM, long-chain lecithins (DMPC, DPPC, and SBPL), and short-chain lecithins (diheptanoyl-PC), stable BR–lipid vesicles are spontaneously formed, which are active in light-energized H^+ translocation. Transmembrane insertion of BR into the lipid bilayer is verified by means of density gradient centrifugation, circular dichroism (CD) measurements, and vectorial H^+ transport. Reconstitution efficiency and general properties of the vesicles depend on the physical and chemical state of the long-chain lecithins. Since the novel method described allows functional transmembrane insertion of BR into lipid vesicles, future work might prove its ability for successful reconstitution of other membrane systems and isolated membrane proteins as well.

Experimental Procedures

Materials. DMPC and DPPC were obtained from Fluka and showed only one spot in thin-layer chromatography. DHPC and SBPL were purchased from Sigma. SBPL was purified by the procedure of Kagawa and Racker (1971). PM isolated from *Halobacterium halobium* S9 showed a single

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[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SBPL, soybean phospholipid(s); DHPC, diheptanoylphosphatidylcholine; CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate.

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band in $NaDodSO_4$–polyacrylamide gel electrophoresis and a ratio of protein aromatic amino acid absorbance at 280 nm to chromophore absorbance at 568 nm of 1.5–1.6 in light-adapted samples.

Vesicle Preparation. For the preparation of vesicles, the respective phospholipids were dissolved in a small volume of chloroform and spread homogeneously as a thin film on the wall of a glass test tube by evaporation of the solvent. Residual traces of solvent were removed under vacuum ($\leq 10^{-3}$ T, 5–15 h) at room temperature. The dried lipid film was hydrated with aqueous 150 mM KCl solution. This step was sometimes assisted by 10-s sonication in a bath-type sonicator (Sonorex RK 100 H, Bendelin Electronic, FRG; 35 kHz). The aqueous dispersion of the long-chain lecithin species (DMPC, DPPC, or SBPL; final concentration after addition of all constituents 20 mM) was mixed with a concentrated aqueous suspension of PM that had been sonicated for 20–420 s prior to mixing to reduce the size of the membrane sheets. Sonication of the PM is not essential. An aqueous 50 mM DHPC solution was added, yielding a final DHPC concentration of 5 mM, and the sample was immediately vortexed. Finally, this lipid–PM mixture was sonicated in the bath sonicator for 30 s. With DMPC and DPPC, but not with SBPL, this sonication step can be omitted. The initial molar phospholipid to BR ratios ranged from 320 to 990. All manipulations were performed at temperatures above the lipid phase transition of the respective long-chain lecithins (e.g., room temperature for SBPL, 30 °C for DMPC, and 44 °C for DPPC). After sonication, the samples were usually incubated at room temperature or 30 °C for 3–8 h and thereafter subjected to density gradient centrifugation (linear 5–40%, w/w, sucrose gradient in 150 mM KCl; 141000*g* for 8 h at 4 °C).

Spectroscopy and H^+ Translocation Assay. Absorption spectra were recorded at room temperature on a Shimadzu UV–vis spectrophotometer UV-240 either in the normal cell compartment or with an integrating sphere attachment. CD measurements were performed with a Jasco J-500A spectropolarimeter using a thermostatically controllable cell of 10-mm path length. Light-induced (500 nm $< \lambda <$ 680 nm, 25 mW/cm^2) pH changes of reconstituted BR–lipid vesicles were monitored with a combined pH electrode in a thermostated vessel equipped with a magnetic stirrer. The pH changes were calibrated by addition of 1-μL injections of 1 mM HCl. Phospholipid concentrations were determined by phosphorus analysis, and the BR concentration was determined both by spectroscopic measurements and by a modified Lowry method corrected for a systematic error as previously described (Rehorek & Heyn, 1979). For electron microscopy, vesicles were stained with either uranyl acetate or ammonium molybdate.

Results and Discussion

Formation of Reconstituted BR–Lipid Vesicles. To monitor the events during vesicle formation, absorption spectra are recorded after each step of the reconstitution procedure. The sample is placed in a 1-mm path-length cuvette in the normal sample compartment far from the photomultiplier. Therefore, the measured spectra are composed of the true absorption by BR (and minor light scattering by PM) and a background due to light scattering of lipid dispersions and vesicles. The large alterations in the light-scattering properties of the sample allow detection of vesicle formation as illustrated in Figure 1. A dispersion of SBPL (20 mM final concentration) is mixed with PM (molar total lipid to BR ratio of 320), and spectrum 1 is recorded. The broad band around 560 nm represents the absorption maximum of the chromoprotein BR and resides on a strong background of light scattering. This is most obvious

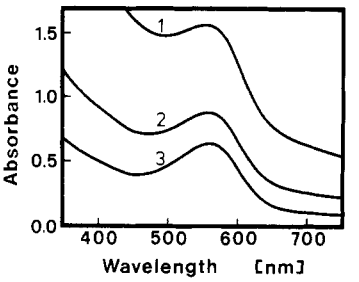

FIGURE 1: Formation of reconstituted SBPL–DHPC–BR vesicles monitored as changes in absorbance and light scattering. (1) Dispersion of SBPL and PM (PM sonicated for 180 s prior to addition); (2) 3 min after addition of aqueous micellar 50 mM DHPC; (3) upon 30-s sonication of (2). Final SBPL–DHPC–BR concentration of 20 mM/5 mM/78 μM, respectively. Room temperature, 1-mm path-length cuvette.

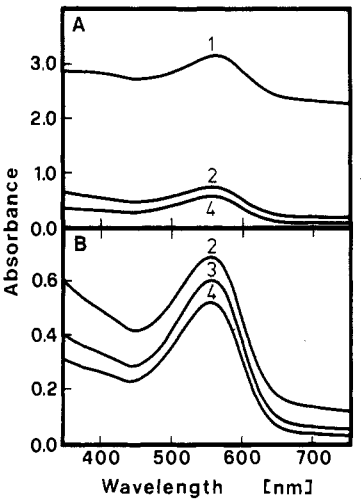

FIGURE 2: Formation of reconstituted DMPC–DHPC–BR vesicles. (1) Dispersion of DMPC and PM (PM sonicated for 30 s prior to addition); (2) 3 min after addition of DHPC at 23 °C; (3) after vortexing (2) at 30 °C and recooling to room temperature; (4) upon 30-s sonication of (3) at 30 °C. All spectra were recorded at room temperature. Spectra 2 and 4 are redrawn in (B) with a more sensitive absorbance scale. Other parameters as described in the legend to Figure 1.

at 750 nm, where BR does not absorb at all and the apparent absorbance is solely due to light scattering by the multibilayer SBPL dispersion. Upon addition of aqueous micellar DHPC (final concentration 5 mM) at room temperature, a pronounced decrease in light scattering of the sample occurs (spectrum 2), much larger than the expected one induced by the 10% dilution involved. Sonication for 30 s leads to a further reduction in light scattering (spectrum 3). The absorption band centered around 560 nm has not changed its position and amplitude significantly, indicating that the spectral properties of BR are not affected by these manipulations. The sample now has an optically clear appearance and is purple in color. If this procedure is repeated with DMPC as the long-chain lecithin, the same events occur; however, the decrease in light scattering upon addition of DHPC is even larger than in the case of SBPL. The DMPC–PM mixture is extremely turbid (Figure 2A, spectrum 1). Addition of DHPC at 23 °C, i.e., at the temperature of the DMPC phase transition, induces a drastic drop in the turbidity (Figure 2A, spectrum 2). If this sample is warmed up to 30 °C and vortexed again, light scattering decreases further (compare spectra 2 and 3 of Figure 2B). The 30-s sonication step leads to an additional but less pronounced reduction in turbidity (Figure 2B, spectrum 4). Experiments

with DPPC (phase transition temperature of 41 °C) substantiate the observation depicted in Figure 2B that DHPC addition has a stronger effect at temperatures above the phase transition of the respective long-chain lecithins. Mixing of DHPC with DPPC at room temperature decreases light scattering by 76% (measured at 750 nm); vortexing at 46 °C leads to a further 23% reduction (data not shown). After being recooled to room temperature, no alterations in light scattering occur. This indicates that the short-chain DHPC interacts more readily with long-chain lecithin bilayers being in the liquid-crystalline than in the gel state. Whereas in case of SBPL (Figure 1) and DMPC (Figure 2) sonication reduces light scattering to various degrees, no effect is observed for DPPC. Control experiments show that in the absence of DHPC no significant change in turbidity is noticeable upon vortexing and 30-s sonication of SBPL–PM, DMPC–PM, and DPPC–PM suspensions. Both, the observed light-scattering changes and electron microscopy (see below) reveal that the short-chain lecithin DHPC facilitates the formation of stable vesicles from aqueous suspensions of preexisting multibilayers of long-chain lecithins (DMPC, DPPC, SBPL) and PM. This is in accordance with the previous report by Gabriel and Roberts (1984) for pure lipid systems. DHPC fails to decrease the turbidity of diphytanoylphosphatidylcholine–PM suspensions. One possible explanation is that the branched phytanoyl chains severely hinder intercalation of DHPC. No satisfactory results are obtained if a dried mixture of SBPL and DHPC is hydrated with an aqueous PM suspension [corresponding to procedure (i) of Gabriel & Roberts (1984)]. Upon vortexing and subsequent 30-s sonication, only a partial decrease in turbidity occurs, and there is no substantial reconstitution of BR. Probably, at least with SBPL, vesicle formation is less effective as with the procedure applied above and might proceed so fast that BR is not inserted into the newly formed lipid bilayer.

Transmembrane Insertion of BR. Whereas the results discussed above clearly demonstrate the ability of DHPC to induce vesicle formation of long-chain lecithins, three different experimental approaches, i.e., density gradient centrifugation, CD, and transport studies, are applied to prove if and to what extent BR is reconstituted into these lipid vesicles. In the linear 5–40% sucrose density gradient, upon centrifugation, reconstituted BR–lipid vesicles are easily identifiable by their color and position in the gradient and clearly separated from pure lipid (at the top of the gradient) and nonincorporated PM (at the bottom). The extent of reconstitution strongly depends on the long-chain lecithin used. In the case of DMPC, complete reconstitution is obtained. Neither free lipid nor nonincorporated PM can be detected in the gradient. The predominant amount of reconstituted BR–lipid vesicles is collected in one broader purple band at lower density. A second, small purple band at slightly higher density contains the rest of the material. The reconstituted vesicles in the main band have an average molar total lipid to BR ratio of 366 and that of the minor band a ratio of 263, as compared to the initial value of 320 for the starting material (PM sonicated for 30 s prior to reconstitution). A similar band pattern of the reconstituted material is observed with DPPC; however, an additional band at the bottom of the gradient appears containing nonincorporated PM. The amount of nonincorporated PM depends on the sonication duration of PM prior to reconstitution and decreases with decreasing PM size. In a sample with an initial lipid to BR ratio of 984 and with PM sonicated for 420 s, the reconstituted material in the band at lower and higher density has molar lipid to BR ratios of 1149 and 627, respectively.

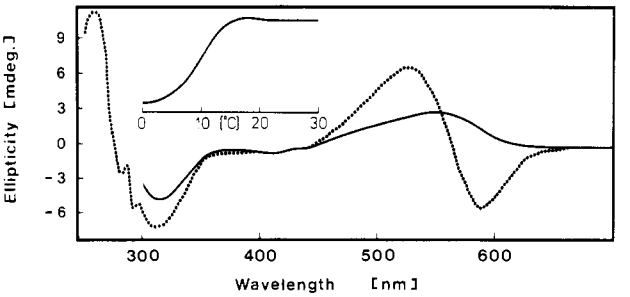

FIGURE 3: Change in the CD spectrum of a suspension of DMPC–DHPC–BR vesicles when the temperature is raised from 3.9 (···) to 25.0 °C (—). Molar lipid to BR ratio of 366. Below 290 nm, both spectra coincide. Insert: Formation of the BR lattice in vesicles as monitored by the temperature dependence of the ellipticity at 585 nm; the temperature was decreased at a rate of 20 °C/h.

Both the band pattern and the actual lipid to BR ratio are very similar in preparations with and without the final 30-s sonication step. In a sample with an initial lipid to BR ratio of 320 and with PM subjected to only 30-s sonication, a considerable proportion of the PM remains nonincorporated, and the majority of the reconstituted DPPC–DHPC–BR vesicles have a lipid to BR ratio of only 715. Reconstitution of BR with SBPL shows a very different behavior. Contrary to the observation with DMPC and DPPC, a large amount of the SBPL is not reconstituted but forms a band close below the surface of the gradient, but well separated from the only reconstituted purple band. Since the majority of PM, but not all, is reconstituted, the determined actual lipid to BR ratio is considerably lower than the initial one. Starting with an initial lipid to BR ratio of 984, the reconstituted vesicle preparation exhibits a ratio of 649 with PM sonicated for only 20 s and of 178 with PM sonicated for 420 s. An initial ratio of 320 and 180-s sonication of PM result in a preparation with a final lipid to BR ratio of 104. Density gradient centrifugation reveals not only the different reconstitution behavior for the three lipid species tested but also the strong irreversible association of BR and lipid. Both CD and transport studies described below exclude the possible argument that the PM might be only encapsulated in the vesicle internal aqueous volume but prove the transmembrane insertion of BR into the lipid bilayer.

A structural feature of the PM is the arrangement of BR in a two-dimensional hexagonal lattice of protein trimers. Due to interactions between retinal chromophores of adjacent immobile BR molecules in the PM lattice, characteristic exciton bands in the CD spectrum between 450 and 650 nm are present. In previous work, it was shown that these features of the CD spectrum can be used to monitor the state of aggregation of BR in PM, detergent solutions, and reconstituted BR–lipid vesicles (Heyn et al., 1975, 1981; Becher & Ebrey, 1976; Dencher & Heyn, 1979; Dencher et al., 1983). The CD spectrum of reconstituted DMPC–DHPC–BR vesicles (collected from the gradient) recorded at 3.9 °C, i.e., far below the transition temperature of the gel to liquid-crystalline phase transition of DMPC, exhibits the same characteristic spectral features as previously described for BR in the PM (Figure 3). This indicates on the one hand that BR itself is not affected by the reconstitution procedure and on the other hand that the BR molecules are arranged in the same spatial orientation as in native PM. Increasing the temperature from below to above the lipid phase transition leads to an alteration of the pair of positive and negative exciton bands characteristic of the hexagonally aggregated state of BR into a broad positive band typical for monomeric BR (Figure 3). Identical CD features and alterations are observed for reconstituted

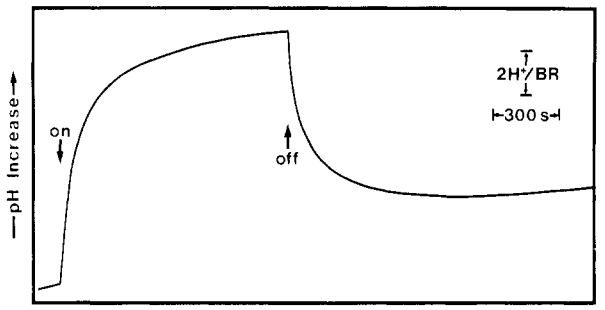

FIGURE 4: Light-induced pH changes generated by SBPL–DHPC–BR vesicles. Arrows depict onset and termination of the illumination period. Vesicles were suspended in 150 mM KCl, initial pH 6.6, 23.0 °C. BR concentration, 1.8 μM; molar lipid to BR ratio, 649.

DPPC–DHPC–BR vesicles at 5 and 44 °C (data not shown). In contrast, native PM and PM incubated with 5 mM DHPC under the same conditions as used for reconstitution, but omitting the long-chain lecithins, reveal no change of the exciton CD spectrum in the temperature range between 2 and 50 °C. On the other hand, the reconstituted SBPL–DHPC–BR vesicles have at all temperatures above 0 °C a CD spectrum composed of the positive band in the visible wavelength range characteristic for monomeric BR, as should be expected for a SBPL phase being in the liquid-crystalline state. The CD results for the DHPC reconstitution procedure can be satisfactorily explained by BR molecules being inserted into the vesicle bilayer membrane composed from long- and short-chain lecithins. Changes in the physical state of this new lipid environment induce the observed reversible aggregation–disaggregation behavior of BR (provided that the molar lipid to BR ratio exceeds approximately 50). Very similar CD data have been previously obtained for BR reconstituted into lipid vesicles by means of detergent dialysis (Dencher & Heyn, 1979; Dencher et al., 1983), detergent dilution (Abdulaev et al., 1984), and freeze–thaw sonication (Casadio & Stoeckenius, 1980) procedures. Since no residual exciton band type features are recognizable in the CD spectra of reconstituted DMPC–DHPC–BR (Figure 3), DPPC–DHPC–BR, and SBPL–DHPC–BR vesicles in the liquid-crystalline state, all BR molecules are incorporated into the vesicle bilayer.

Illumination of a suspension of SBPL–DHPC–BR vesicles with light absorbed by BR's chromophore retinal results in a large reversible alkalization of the external medium (Figure 4). The steady-state value corresponds to a net inward translocation of about 11 H^+/BR. The light-induced alkalization is inhibited by protonophores or solubilization of the vesicle membrane by the detergent Triton X-100. Similar steady-state values of protons translocated per BR have been previously reported for BR reconstituted into SBPL vesicles by means of detergent dialysis and detergent dilution techniques (Dencher & Heyn, 1979; Racker et al., 1979; Abdulaev et al., 1984). This efficient light-induced vectorial H^+ transport confirms transmembrane insertion of BR and furthermore illustrates that the DHPC reconstitution procedure is not deleterious for the proton pump mechanism of this chromoprotein. Control experiments show that the presence of the short-chain lecithin is a necessary prerequisite for BR's reconstitution. Upon illumination of an aqueous SBPL–PM dispersion, only a small decrease in pH corresponding to an H^+ release of $\leq$0.08 H^+/BR occurs, which is replaced by a small increase in pH corresponding to an H^+ uptake of $\leq$0.16 H^+/BR upon 30-s sonication of the dispersion. Even after prolonged sonication, the light-induced pH changes are considerably smaller (0.9 H^+/BR and 1.5 H^+/BR upon 180- and 600-s sonication, respectively) than those observed with material sonicated only 30 s in the presence of DHPC (11 H^+/BR, Figure 4). The approximately 100-fold increase in vectorial transport activity at otherwise identical conditions demonstrates best the efficiency of DHPC to reconstitute BR into SBPL vesicles. From the direction of light-induced proton translocation, i.e., into the vesicles, which is opposite to that observed for intact halobacteria, a net inside-out orientation of BR in the SBPL–DHPC vesicles has to be concluded. This inside-out orientation of BR is commonly obtained with other reconstitution procedures as well. With DMPC–DHPC–BR and DPPC–DHPC–BR vesicles, both below and above the lipid phase transition temperature, light-induced fast acidification occurs corresponding to $\leq$1.3 H^+/BR released into the external medium. H^+ ionophores and detergent treatment abolish part of the signal. The net outside-out orientation of the BR molecules deduced from the direction of the pH signal is in accordance with the outside-out orientation of the majority of the BR molecules determined by selective proteolysis and subsequent gel electrophoresis (P. Burghaus, unpublished observation). The fact that the net orientation of the BR molecules depends on the long-chain lecithins used is an interesting feature of this reconstitution procedure; with SBPL, a net inside-out orientation of BR is produced, whereas with DMPC and DPPC a net outside-out orientation is obtained. To what extent this is related to the observed differences in the vesicle size (see below) has to be elucidated. The preferential orientation of BR in reconstituted vesicles is also determined by the degree of dissociation of the lipid phosphate groups (Happe et al., 1977). Acidifying BR vesicles are formed with partial neutralized polar head groups.

The less efficient proton transport activity of DMPC–DHPC–BR and DPPC–DHPC–BR vesicles as compared with the corresponding SBPL vesicles is an inherent property of the former long-chain lecithins since the same dependency is found for detergent dialysis vesicles lacking DHPC (Dencher & Heyn, 1979).

Properties of Reconstituted BR–Lipid Vesicles. An important advantage of the reconstitution procedure described is, besides its easiness and quickness, the fact that the membrane protein is not damaged. (It is worth mentioning that sonication during reconstitution is only necessary for SBPL but not for the two other long-chain lecithins used, i.e., DMPC and DPPC. Furthermore, the duration of sonication could be even reduced.) The most rigorous test is the direct incubation of PM with 5 mM DHPC at room temperature for 48 h (molar BR:native PM lipids:DHPC ratio of 1:7:64). As compared to the absorption spectrum of untreated PM, neither the amplitude and position of the chromophore absorption band at 568 nm nor the amplitude and position of the aromatic amino acid region around 280 nm are changed. Also, the bands in the CD spectrum of BR reflecting chromophore–chromophore interactions (exciton coupling bilobe), intrinsic retinal–protein interactions (at 318 nm), and integrity and environment of the aromatic amino acids (250 nm $< \lambda <$ 300 nm) are not affected by treatment with DHPC. These unchanged features and the considerable H^+ transport activity (Figure 4) exclude any harmful interactions of DHPC with BR itself. Upon incubation with DHPC, neither the density of the PM changes nor any mobility of BR is induced. Absorption (Figures 1 and 2) and CD (Figure 3) spectra of reconstituted BR also indicate no harmful effect of the reconstitution procedure. Small differences between BR in the PM and reconstituted BR can be explained by interactions with the foreign long-chain lipid species and monomerization of BR induced by the high lipid to protein ratio and the liquid-

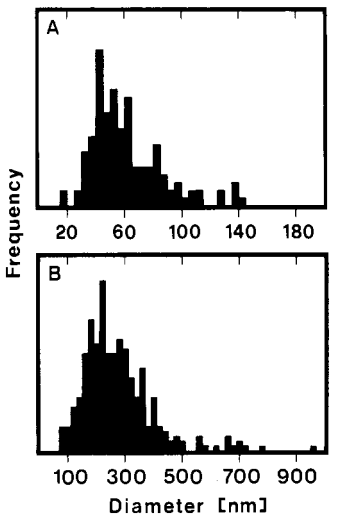

FIGURE 5: Size distribution of SBPL–DHPC–BR (A) and DMPC–DHPC–BR (B) vesicles as determined by electron microscopy. Molar lipid to BR ratios of 649 (A) and 366 (B). Number of measured vesicles: 134 (A), 307 (B).

crystalline state of the lipid phase, as previously shown (Casadio & Stoeckenius, 1980; Dencher et al., 1983). The integrity of the vesicles and the stability of BR, as deduced from the H^+ transport assay and spectroscopy, change only slightly during 9 days storage at 4 °C. The larger decline in stability of SBPL–DHPC–BR vesicles (14%) as compared to DMPC–DHPC–BR and DPPC–DHPC–BR vesicles (<5%) is in line with similar observations with other reconstitution procedures for SBPL. Although DHPC does not affect BR itself, it has an effect on the physical parameters of the lipid phase, as can be expected for such a short-chain component at this relatively high concentration (20 mol % of total lipid). In the insert of Figure 3, the temperature dependence of the ellipticity at 585 nm, the wavelength of maximal difference between the CD spectra of monomeric and aggregated BR, is plotted. This CD transition curve that monitors the reversible crystallization of BR induced by the lipid phase transition is characterized by a midpoint at 9.9 °C for DMPC–DHPC–BR vesicles (Figure 3) and 31.5 °C for the respective vesicles reconstituted with DPPC. For BR in a pure DMPC and DPPC bilayer, however, transition midpoint temperatures of 15–16 and 34.5 °C, respectively, are observed (Dencher & Heyn, 1979; Heyn et al., 1981; Dencher et al., 1983). Furthermore, in DMPC–DHPC–BR vesicles, a different cleavage pattern of BR by chymotrypsin is found as compared to BR in PM and in reconstituted DMPC vesicles (P. Burghaus, unpublished result), indicating a thinner or/and perturbed lipid bilayer. On the other hand, at least with SBPL–DHPC–BR vesicles, the permeability of the lipid bilayer for protons and/or for the counterions potassium and chloride is not affected by the presence of DHPC. As compared with SBPL–BR vesicles reconstituted by a detergent dialysis procedure, the permeability is even less [see Figure 2b of Dencher & Heyn (1979)]. A decrease in the relative proportion of DHPC might prevent or at least reduce the described effect on the bilayer. Decreasing the molar DHPC to SBPL ratio from the usual value of 0.25 to 0.05, however, results in a considerably smaller extent of vesicle formation.

The long-chain lecithins seem to determine the size of the reconstituted vesicles. Figure 5A,B shows the size distribution of SBPL–DHPC–BR and DMPC–DHPC–BR vesicles obtained by electron microscopy (carried out by R. Groll, Fritz-Haber-Institute of the Max-Planck-Gesellschaft, Berlin, FRG). The majority of the SBPL–DHPC–BR vesicles have diameters between 35 and 100 nm, whereas the DMPC–DHPC–BR vesicles are much larger, ranging from 100 to 450 nm in diameter. Vesicles as large as 1 μm are present in the latter sample. A smaller size of SBPL vesicles as compared to diphytanoyl-PC vesicles is also observed for pure lipid vesicles produced by sonication (Grzesiek & Dencher, 1985). For DHPC–DPPC vesicles, a particle size of 61 nm is reported by Gabriel and Roberts (1984). These authors also show that the vesicles formed spontaneously from short- and long-chain lecithins are unilamellar. The clarity of the vesicle suspensions (Figures 1 and 2), the extent of proteolytic digestion of BR, the relatively fast rates of light-induced H^+ translocation and especially of the passive back-diffusion of protons upon termination of illumination (Figure 4), and the electron microscopic data indicate that the reconstituted BR–lipid vesicles are predominantly unilamellar as well.

Recently, during the editorial preparation of this report, another promising reconstitution procedure has been published that utilized the fusogen myristate and cooling to temperatures below the gel to liquid-crystalline phase transition (Scotto & Zakim, 1985).

Conclusion

Mixing aqueous suspensions of long-chain lecithins (DMPC, DPPC, SBPL) with the short-chain lecithin DHPC (20 mol % total lipid) in the presence of PM leads to the spontaneous formation of reconstituted BR–lipid vesicles. BR is inserted into the lipid bilayer and is highly active as a light-energized proton pump. Reconstitution and properties of the reconstituted vesicles strongly depend on the chemical and physical state of the long-chain lecithins. Since the procedure is easy, fast, and particularly has no harmful effect on the membrane protein, it might be a method of choice for functional reconstitution of other membrane systems (in particular, membranes highly enriched in one protein species, e.g., rhodopsin in the disk membrane of photoreceptors, acetylcholine receptors in membranes of electric organ or synapse, etc.) as well as isolated purified membrane proteins (e.g., in the presence of very small amounts of detergents).

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References

Abdulaev, N. G., Dencher, N. A., Dergachev, A. E., Fahr, A., & Kiselev, A. V. (1984) *Biophys. Struct. Mech. 10*, 211–227.

Becher, B., & Ebrey, T. G. (1976) *Biochem. Biophys. Res. Commun. 69*, 1–6.

Casadio, R., & Stoeckenius, W. (1980) *Biochemistry 19*, 3374–3381.

Dencher, N. A., & Heyn, M. P. (1979) *FEBS Lett. 108*, 307–310.

Dencher, N. A., Kohl, K.-D., & Heyn, M. P. (1983) *Biochemistry 22*, 1323–1334.

Eytan, G. D. (1982) *Biochim. Biophys. Acta 694*, 185–202.

Gabriel, N. E., & Roberts, M. F. (1984) *Biochemistry 23*, 4011–4015.

Grzesiek, S., & Dencher, N. A. (1985) *Biophys. J. 47*, 274a.

Happe, M., Teather, R. M., Overath, P., Knobling, A., & Oesterhelt, D. (1977) *Biochim. Biophys. Acta 465*, 415–420.

Heyn, M. P., & Dencher, N. A. (1982) *Methods Enzymol. 88*, 31–34.

Heyn, M. P., Bauer, P.-J., & Dencher, N. A. (1975) *Biochem. Biophys. Res. Commun. 67*, 897–903.
Heyn, M. P., Cherry, R. J., & Dencher, N. A. (1981) *Biochemistry 20*, 840–849.
Hwang, S.-B., & Stoeckenius, W. (1977) *J. Membr. Biol. 33*, 325–350.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.
Montal, M., Darszon, A., & Schindler, H. (1981) *Q. Rev. Biophys. 14*, 1–79.
Racker, E. (1979) *Methods Enzymol. 55*, 699–711.
Racker, E., Violand, B., O'Neal, S., Alfonzo, M., & Telford, J. (1979) *Arch. Biochem. Biophys. 198*, 470–477.
Rehorek, M., & Heyn, M. P. (1979) *Biochemistry 18*, 4977–4983.
Rigaud, J. L., Bluzat, A., & Buschlen, S. (1983) *Biochem. Biophys. Res. Commun. 111*, 373–382.
Scotto, A. W., & Zakim, D. (1985) *Biochemistry 24*, 4066–4075.
Van Dijck, P. W. M., Nicolay, K., Leunissen-Bijvelt, J., Van Dam, K., & Kaptein, R. (1981) *Eur. J. Biochem. 117*, 639–645.