Reconstitution of Bacteriorhodopsin Vesicles with *Halobacterium halobium* Lipids

EFFECTS OF VARIATIONS IN LIPID COMPOSITION*

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Reconstitution of bacteriorhodopsin into vesicles has been studied using individual and defined mixtures of fractionated *Halobacterium halobium* polar lipids. The lipids varied greatly in their ability to form vesicles that translocated protons and showed stimulation by valinomycin. Thus, phosphatidylglycerol phosphate gave mainly lipid-protein aggregates, while glycolipid sulfate gave mainly functional vesicles. Addition of glycolipid sulfate to phosphatidylglycerol phosphate promoted vesicle formation. Reconstitutions performed with different combinations of the purified lipids showed: 1) that vesicles with maximal proton pumping are obtained with a mixture of phosphatidylglycerol phosphate and glycolipid sulfate at a ratio close to that found in the purple membrane; 2) increasing the concentration of glycolipid sulfate in vesicles increases the stimulation of proton translocation by valinomycin; 3) phosphatidylglycerol phosphate cannot replace glycolipid sulfate or phosphatidylglycerol phosphate; and 4) vesicles containing sulfated polar lipids only are inferior to those in which lipid combinations containing phosphatidylglycerol phosphate are used.

Bacteriorhodopsin, the only protein present in the purple membrane patches of the cell membrane of halophilic bacteria, catalyzes a light-driven vectorial translocation of protons and thus generates a transmembrane electrochemical gradient. With the aim of studying structure-function relationships in bacteriorhodopsin and to investigate the role of endogenous lipids in the proton translocation process, we have previously described the complete delipidation of the purple membrane (2). Further, reconstitution of the lipid-free bacteriorhodopsin with exogenous lipids to form efficient proton-translocating vesicles was accomplished. In a more recent report, we showed that delipidated bacteriorhodopsin can be reconstituted with the polar lipids from *Halobacterium halobium* (3). While the resulting vesicles, like those prepared with exogenous lipids, had the inside-out orientation, they showed several important characteristics that were different, such as lower ion permeability and homogeneity with regard to bacteriorhodopsin and lipid content.

Purple membrane contains a number of polar lipids. The main lipids are phosphatidylglycerol phosphate and glycolipid sulfate, but, in addition, phosphatidylglycerol sulfate and phosphatidylglycerol, are present as minor components (4, 5). These lipids are all negatively charged and they all contain the phytanyl group, as the hydrocarbon chain which is connected to glycerol hydroxyl groups in ether linkages (6). Another notable feature is that GLS and PGS are present only in the purple membrane (4). There are several intriguing questions in regard to the role and specificity of the polar lipids that participate in the formation of the highly organized purple membrane. Thus, why are there several lipids differing only in the nature of the negatively charged polar head groups? Do lipids carrying an extra phosphomonoester group, such as PGP, differ in their function from those that have the extra sulfate group? What is the contribution or function of the trisaccharide moiety that is present in GLS? Is there a balance that must be maintained between the different lipids for the structural stability of the purple membrane. In our present study, we have performed reconstitution experiments using purified individual purple membrane lipids and mixtures containing defined ratios of different polar lipids. Wide variations were observed between the individual lipids in the ability to form vesicles and to translocate protons. GLS seemed to be important both for the formation of vesicles as well as for the low ion permeability reported previously (3) in vesicles obtained from reconstitution of bacteriorhodopsin with the total endogenous lipids. Furthermore, a mixture of PGP and GLS at a ratio close to that found in vivo appeared to create the best lipid environment for efficient proton translocation by bacteriorhodopsin.

MATERIALS AND METHODS

These were all as described previously (3, 7) except for the following:

Isolation of Polar Lipids from *H. halobium*—Total polar lipids were obtained as described by Hancock and Kates (8) with minor modifications reported previously (3, 7). The polar lipids were separated essentially according to the method of Kates and co-workers (8, 9) by preparative thin layer chromatography in two solvent systems. System A consisted of chloroform/acetic acid (90%/methanol (15:10) and system B consisted of chloroform/methanol/ammonium hydroxide (50:50:4). The separated lipids were eluted from the silica gel with an acidic Bligh-Dyer solvent system described by Kates and Dero (9) and stored in chloroform at −20 °C. The purity of the isolated lipids was checked by thin layer chromatography in three different systems.

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1 The abbreviations used are: GLS, glycolipid sulfate; PGS, phosphatidylglycerol phosphate; PGP, phosphatidylglycerol phosphate; 2,3-di-O-phytanyl-1-O-[galactosyl-3'-sulfate-β(1',6')-mannosyl-a(1',2')-glucosyl-a(1',1')]-sn-glycerol; HhPl; H. halobium polar lipids; PGS, phosphatidylglycerol sulfate; PGP, phosphatidylglycerol sulfate; and PGS, phosphatidylglycerol sulfate.
**RESULTS**

**Proton Translocation by Bacteriorhodopsin Vesicles Reconstituted with Individual and Combinations of Endogenous Lipids**

**Purified Individual Lipids—**Reconstitution of bacteriorhodopsin with PGP, GLS, and PGS resulted in each case in vesicles that translocated protons, the efficiency ranging between 25 to 60% of that shown by HhPL vesicles (Table I). The extent as well as the rate of proton uptake was increased by valinomycin (Table I) as was previously observed with bacteriorhodopsin vesicles reconstituted with HhPL (3). The stimulation by valinomycin was particularly pronounced in GLS proteoliposomes. Bacteriorhodopsin vesicles reconstituted with PG showed a remarkably high proton-translocating activity and stimulation by valinomycin was the highest among the vesicles reconstituted with an individual lipid (Table I).

**PGP and GLS in Different Ratios—**Fig. 1A shows the proton translocation properties of vesicles obtained by reconstitution of bacteriorhodopsin with varying ratios of PGP and GLS. Maximal extent of proton translocation (approximately 52H+/- bacteriorhodopsin) was observed when the ratio of PGP to GLS was between 4:2 and 2:4 (w/w; Fig. 1A). However, the stimulation of proton translocation by valinomycin was almost twice as high at a ratio of PGP to GLS of 2:4 compared to that obtained with a PGP/GLS ratio of 4:2. Maximal stimulation (12-fold) by valinomycin was observed at a PGP/GLS ratio of 1:5 (w/w; Fig. 1A). The rate of proton translocation and its stimulation by valinomycin (Fig. 1B) was found to follow a pattern similar to that of the extent of proton translocation shown in Fig. 1A.

**Varying Ratios of PGP and GLS with a Constant Amount of PGS or PG—**To simulate the lipid composition of the purple membrane a low constant amount of PGS or PG was included in reconstitution of bacteriorhodopsin with varying ratios of PGP to GLS. The pattern observed in the presence of PGS (Fig. 2) was very similar to that obtained in its absence within different solvent systems: A, B, and chloroform/methanol/water (50:50:5). Each individual lipid revealed a single spot in all solvent systems after spraying with a phosphate reagent (10) or with 2 M NH₄SO₄ followed by charring. The lipids were quantitated from the phosphate content (11) or hexose content (12) using sucrose as a standard.

**Reconstitution of Bacteriorhodopsin—**A ratio of lipid to protein of 40:1 (w/w) was employed in reconstitution of delipidated bacteriorhodopsin by the cholate dialysis method (3, 7). In general, the recovery of the chromophore in the various vesicle preparations was close to 100%. Proton translocation activity of bacteriorhodopsin-containing vesicles (20 to 100 nm) was assayed in 2 ml of 0.15 M NaCl in the absence and in the presence of 2 μM valinomycin. The proton translocation properties of the vesicles were fully retained after 3 months storage in the dark at 4 °C.

**TABLE I**

| Lipids used | Initial rate | Stimulation by | Extent | Stimulation by |
|-------------|--------------|----------------|--------|----------------|
|             | +Val | -Val | +Val | -Val | +Val | -Val |
| PGP         | 1.3  | 2.5  | 1.9  | 6.3  | 16.5 | 2.6  |
| GLS         | 0.9  | 3.4  | 3.6  | 2.6  | 10.8 | 4.2  |
| PGS         | 1.7  | 2.9  | 1.7  | 5.6  | 17.8 | 3.2  |
| PG          | 2.1  | 7.9  | 3.8  | 5.1  | 38.9 | 7.6  |
| HhPL        | 1.5  | 5.6  | 3.6  | 5.8  | 43.1 | 7.4  |

*Expressed as nanomoles H per nmol bacteriorhodopsin per s.

**Fig. 1.** Extent (A) and initial rate (B) of proton translocation from vesicles obtained from reconstitution of bacteriorhodopsin (BR) with various ratios of PGP to GLS. Reconstitution of delipidated bacteriorhodopsin with PGP and GLS was performed by the cholate dialysis method as described under "Materials and Methods." The light-dependent proton translocation by the vesicles was assayed in the absence and presence of valinomycin (2 μM).

**Fig. 2.** Extent (A) and initial rate (B) of proton translocation by vesicles obtained by reconstitution of bacteriorhodopsin (BR) with various ratios of PGP to GLS and a constant amount of PGS. Experimental conditions were as described in Fig. 1 and under "Materials and Methods."
The presence of PG in PGP-GLS proteoliposomes did not affect the stimulation of proton translocation by valinomycin (Fig. 3). The rate of proton translocation was essentially constant at the different lipid ratios tested (Fig. 3B). Also, the extent of proton translocation was constant over a broad range although it decreased considerably with increasing amounts of GLS (PGP/GLS/PG = 1:5:1) (Fig. 3A).

**PGP and PGS, in Different Ratios**—Reconstitution of bacteriorhodopsin with varying ratios of PGP and PGS resulted in preparations that gave relatively low extent of proton translocation (Fig. 4A) whereas the maximal rate of proton translocation (Fig. 4B) did not differ significantly from those given by other proteoliposomes. However, the stimulation of both the extent and rate of proton translocation by valinomycin (Fig. 4) were much lower than those observed in PGP-GLS vesicles (Fig. 1).

**PGS and GLS, in Different Ratios**—The extent of proton translocation by bacteriorhodopsin reconstituted with various ratios of PGS to GLS (Fig. 5A) was found to be lower than that observed with PGP-GLS vesicles. Also the stimulation by valinomycin (Fig. 5A) was likewise low. Although the rate of proton translocation (Fig. 5B) was similar to that observed with PGP-GLS proteoliposomes, the stimulation by valinomycin was considerably lower.

**Sucrose Density Gradient Fractionation of Different Bacteriorhodopsin Vesicle Preparations**

Bacteriorhodopsin vesicles reconstituted with H. halobium GLS alone, PGP alone, PGP and GLS mixtures were subjected to sucrose density gradient centrifugation and the fractions analyzed for bacteriorhodopsin content and proton translocation activity. *H. halobium* lipid proteoliposomes, which contain PGP and GLS in a ratio of approximately 4:2 (w/w), fractionated into a major highly active vesicle population and a minor aggregated fraction with higher density and low proton-translocating activity (Fig. 6A). These results are similar to those...
Reconstitution of Bacteriorhodopsin with *H. halobium* Lipids

Table II

| Vesicle preparation | Density | Initial rate | Stimulation by Val | Extent | Stimulation by Val |
|---------------------|---------|--------------|--------------------|--------|--------------------|
| HhP*                | 1.069   | 4.4          | 4.8               | 32.5   | 8.8                |
| PGP                 | 1.050   | 4.6          | 2.6               | 32.6   | 6.0                |
| GLS                 | 1.091   | 3.9          | 4.3               | 10.0   | 5.5                |
| PGP/GLS*            | 1.053   | 5.3          | 3.7               | 32.9   | 7.0                |
| PGP/GLS**           | 1.065   | 4.2          | 3.4               | 40.3   | 12.9               |

*For vesicles from total HhPL lipids and PGP + GLS at ratios of 5:1 and 2:4 the highest proton translocation per bacteriorhodopsin was observed in fractions of slightly lower densities in agreement with previous findings (3).*

The gradual increase in the relative content of GLS (Fig. 6) which probably was due to the inherent higher density of this lipid.

In Table II are summarized the proton-translocating properties of the different bacteriorhodopsin-containing vesicle preparations after fractionation on sucrose density gradients. As expected, removal of the turbid lipid-protein aggregates from bacteriorhodopsin-PGP vesicles resulted in an approximately 2-fold increase in both the rate and the extent of proton translocation (Tables I and II). The stimulation by valinomycin of the extent, but not of the rate, of proton translocation also increased on fractionation although it never reached a level similar to that found for vesicles prepared from PGP and GLS (2:4, w/w, Table II). As described in Fig. 1, PGP-proteoliposomes containing a small amount of GLS preparation (PGP/GLS, 5:1 w/w) showed an increased valinomycin stimulation of proton translocation but this was only slightly increased upon fractionation on a sucrose gradient (Table II). Similar results were obtained from preparations of PGP-GLS (2:4, w/w) proteoliposomes which contained only minor amounts of protein-lipid aggregates. However, these vesicles exhibited a much higher stimulation of proton translocation by valinomycin relative to that of the PGP-GLS (5:1 w/w) preparations. These results indicated that the addition of GLS to PGP-proteoliposomes enhanced vesicle formation which reached a maximal level at a PGP/GLS ratio of approximately 4.2 (w/w). Further increase in the content of GLS in PGP-proteoliposomes resulted in a decrease in ion permeability of the membrane as shown by the increased stimulation of proton translocation by valinomycin.

**DISCUSSION**

As pointed out above, the polar lipids of purple membrane differ only in their head groups. However, GLS, which is exclusively found in the purple membrane (4), is the only lipid that has been reported to form osmotically active liposomes (13). The present study indicates that the presence of bacteriorhodopsin clearly promotes the formation of proton-translocating vesicles (see e.g. Table I). However, the lipids vary widely in their ability to form vesicles. Thus, reconstitution of bacteriorhodopsin with PGP only gave mainly protein-lipid aggregates as judged from fractionation on sucrose...
density gradients (Fig. 6C) and on Sepharose CL 4B columns (data not shown). On the other hand, the use of GLS in reconstitution gave a rather homogeneous vesicle population (Fig. 6B). GLS appeared to be important for vesicle formation since addition of this lipid to reconstitutions of bacteriorhodopsin with PGP, up to a PGP-GLS ratio of 4:2, mainly gave vesicles rather than lipid-protein aggregates (Fig. 6).

The properties reported previously (3) for bacteriorhodopsin vesicles reconstituted from the endogenous lipids would appear, from the present results, to be derived from the presence of GLS as well as of PGP in the membrane. Thus, vesicles with maximal proton-translocating activity were obtained from reconstitutions of bacteriorhodopsin with PGP and GLS at a ratio between 4:2 to 3:3 (w/w; Fig. 1) which is close to the relative composition of these lipids in the purple membrane (4). The proton translocation by these vesicles was strikingly increased in the presence of valinomycin. An even higher stimulation by the ionophore was observed in vesicles for bacteriorhodopsin since a mixture of the two sulfated membrane was obtained in experiments in which GLS was head groups of the individual lipids have characteristic effects on orientation of the protein in vesicles has recently been confirmed (17). With the same technique an identical orientation of bacteriorhodopsin was found in vesicles reconstituted with all of the individual lipids of purple membrane with the exception of PGP-proteoliposomes where only 40 to 60% of the carboxyl termini were accessible to antibody binding probably due to the large amounts of lipid-protein aggregates observed. While factors determining this inside out orientation of bacteriorhodopsin have yet to be clarified, the curvature of the bilayer in the small vesicles and/or the large excess of lipids present, compared to the situation in purple membrane, could be possibilities.

There are several aspects concerning the role of the unusual lipids in purple membrane that remain poorly understood. Thus, e.g., what is the function of the asymmetric distribution of the purple membrane lipids, i.e., why is GLS exclusively present on the outer surface (16)? Do these lipids have a specific role in the maintenance of the crystalline trimeric structure of bacteriorhodopsin in purple membrane (17)?

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B Höjeberg, C Lind and H G Khorana

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