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DIRECTION OF PROTON TRANSLOCATION IN PROTEOLIPOSOMES FORMED FROM PURPLE MEMBRANE AND ACIDIC LIPIDS DEPENDS ON THE pH DURING RECONSTITUTION

MONIKA HAPPE[a], RON M. TEATHER[a], PETER OVERATH[a], ANSGAR KNOBLING[b] and DIETER OESTERHELT[b]

[a]*Max-Planck-Institut für Biologie, D74 Tübingen, and* [b]*Institut für Biochemie der Universität Würzburg, D87 Würzburg (G.F.R.)*

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Summary

The reconstitution of proton pumping activity in proteoliposomes formed by brief sonication of purple membrane and lipid dispersions was studied as a function of pH. Proteoliposomes reconstituted using cardiolipin showed light-dependent proton extrusion when formed at a pH below 2.75 and proton uptake when formed above pH 2.75. Several other acidic lipids including halobacterial lipids behaved similarly. The experiments suggest that the degree of dissociation of the lipid phosphate groups determines the preferential orientation of bacteriorhodopsin in reconstituted proteoliposomes.

The purple membrane of *Halobacterium halobium* is a specialized part of the cell membrane and contains a single protein, bacteriorhodopsin (75% by weight), and 25% lipids [1]. Total lipid extracts are composed of about 60% phospholipids (phosphatidylglycerolphosphate and its sulfate analogue) and 30% glycolipids [2—4]. In vivo, bacteriorhodopsin catalyzes light-driven proton translocation from the interior of the cell to the outside medium [5,6]. In contrast, proteoliposomes reconstituted in vitro from purple membrane sheets and lipid vesicles show light-dependent proton uptake [7—10]. The preferred orientation of bacteriorhodopsin in the membrane of the reconstituted vesicles thus appears to be opposite to that in whole cells. This view is supported by freeze-etch electron microscopy [11].

In the reconstitution method of Racker [7], purple membrane and lipid vesicles are dispersed in 0.15 M KCl and sonicated. Proton uptake is measured after adjustment of the pH to 6.2. As mentioned by Racker and Hinkle [9], it is possible that the preferred orientation of bacteriorhodopsin in the reconstituted membrane might be altered by changing the conditions of reconstitution. In this paper we report a systematic study on the effect of pH on the preferential orientation of bacteriorhodopsin in the reconstituted vesicles

using a variety of phospholipids including halobacterial lipids.

Our experimental procedure is similar to that of Racker [7] except that the pH was carefully controlled during reconstitution. Phospholipids (1—5 ml at a concentration of 10 mg/ml) were sonicated under an N_2-atmosphere in 0.15 M KCl at 20°C for 30 min with a Branson Sonifier at an output of 25 W. The dispersions were then diluted ten-fold with 0.15 M KCl and adjusted to the desired pH with 1 M HCl. 1 ml of purple membrane suspension [12] containing 0.62 mg of protein in 0.15 M KCl was adjusted to the same pH and added to 5 ml of the diluted lipid dispersion. The samples were then sonicated for an additional 30 s as described above. This short sonication time was dictated by the tendency of bacteriorhodopsin to bleach under acid conditions. Immediately after this second sonication, the pH was adjusted to 6.0 with 1 M NaOH. The procedure thus gives constant starting conditions for the assay of light-dependent pH changes. While bacteriorhodopsin is stable at pH < 3.0 in the intact purple membrane, partial bleaching (⩽40%) at acid pH could not be prevented during incorporation into liposomes. It is important to notice that the reconstituted samples could be stored at room temperature for several hours without a change in the direction or extent of the proton translocating activity.

Cardiolipin isolated from *Escherichia coli* cells proved to be the most convenient lipid for describing the main observations of this report. Fig. 1 (upper trace) shows that proteoliposomes reconstituted at pH 2.5 show a light-dependent acidification of the medium. When prepared at pH 3.5 (lower trace), an alkalinization of the medium is observed. Both effects are reversed when the light is shut off. Addition of the uncoupler carbonyl cyanide 3-chlorophenylhydrazone effectively inhibits the light-dependent pH changes in both preparations. In addition, strong inhibition was observed with the K^+/H^+-antiporter

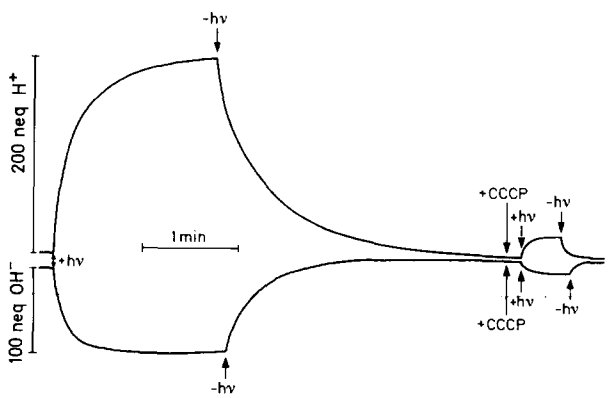

Fig. 1. Light dependent pH changes by proteoliposomes reconstituted from cardiolipin and purple membrane. Cardiolipin was purified from total lipid extracts of *E. coli* cells by silicic acid chromatography. Purple membrane and a dispersion of cardiolipin were sonicated together at pH 2.5 or pH 3.5 as described in the text. After adjustment of the pH to 6.0, samples were transferred to a stirred 5-ml cell with a glass electrode (Einstab Messkette, Ingold, Frankfurt/Main) connected to a Radiometer Copenhagen pH meter. Light from a 150 W halogen lamp was filtered with an orange filter (Schott 0G4) and focused on the cell with auxiliary lenses. pH changes were standardized by addition of 10^{-2} M NaOH or HCl. The upper trace shows the time course of light-dependent proton extrusion by proteoliposomes prepared at pH 2.5. The lower trace shows light-driven proton uptake by proteoliposomes prepared at pH 3.5. Carbonylcyanide 3-chlorophenylhydrazone (CCCP) was added to a final concentration of 10^{-4} M.

nigericin at a concentration of 0.2 μg/ml. These experiments are most readily explained by the assumption that proteoliposomes prepared at pH 2.5 catalyze an active proton extrusion while those prepared at pH 3.5 show an active proton uptake.

The extent of proton uptake or extrusion varies considerably from experiment to experiment (compare Figs. 1 and 2a). However, the direction of net proton flux as a function of pH during the reconstitution is always reproducible. This is evident from Fig. 2a which shows three independent experiments using cardiolipin. At a pH < 2.75 acidifying preparations are obtained, with a maximum in activity at pH 2.5. At a pH > 2.75 alkalinizing preparations are obtained, with a maximum activity at pH 3.5. When prepared at pH $\simeq 6.0$, proteoliposomes reveal no light-dependent pH changes under these conditions. At this pH extended sonication of the mixture is required for significant uptake to occur.

Fig. 3 shows the extent of proton extrusion (upper curve) or proton uptake (lower curve) by proteoliposomes reconstituted with varying amounts of cardiolipin at pH 2.5 or pH 3.5, respectively. Under both conditions, no further increase in activity is observed above a cardiolipin content of about 1.2 mg per assay. Sucrose gradient centrifugation of samples reconstituted under standard conditions showed that the recombinant proteoliposomes had a molar ratio of lipids to protein of 31 when formed at pH 2.5 and 37 when formed at pH 3.5.

The interpretation of light-driven pH changes as being caused by the active movement of protons obviously requires that at least some of the proteoliposomes form closed structures of low passive permeability. Proteoliposomes were reconstituted with cardiolipin at pH 3.5 or 2.5 in the presence of [^{3}H]lactose and passed over a Sephadex G50 column. Both samples gave

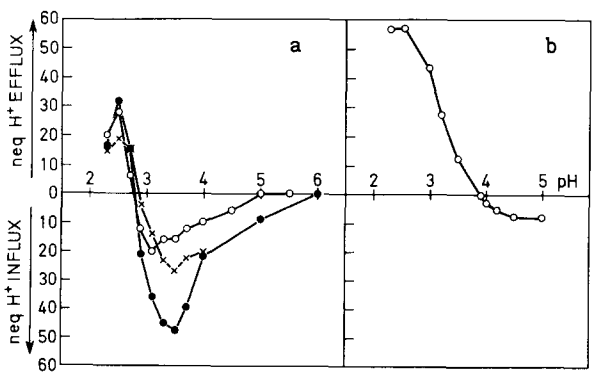

Fig. 2. Dependence of direction of proton flux in proteoliposomes on pH during reconstitution with cardiolipin (a) or halobacterial lipids (b) and purple membrane. (a) Proteoliposomes were reconstituted from cardiolipin dispersions and purple membrane as described in the text. Each assay contained 4.2 mg cardiolipin and 0.5 mg bacteriorhodopsin in 0.15 M KCl. The plateau level of proton uptake or extrusion upon illumination (cf. Fig. 1) as a function of pH during reconstitution is shown for three independent experiments. (b) *H. halobium* lipids (1—5 ml, 10 mg/ml) were sonicated in 0.15 M KCl at 0°C for 30 min in a Branson Sonifier at a power output of 40 W. 0.9 ml of this dispersion was mixed with 0.05 ml purple membrane suspension (0.62 mg protein) and 0.35 ml 0.15 M KCl. After adjustment to the desired pH with 1 M HCl the samples were sonicated for 1 min as described above and immediately thereafter adjusted to pH 6.5 with 1 M KOH. The samples were diluted to a final volume of 5 ml with 0.15 M KCl. Light-dependent pH changes were measured as described in the legend to Fig. 1.

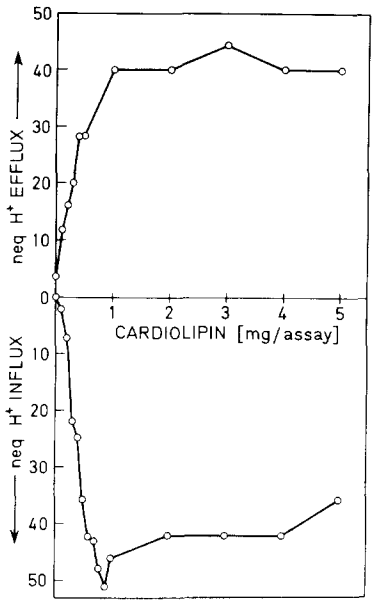

Fig. 3. Extent of proton flux as a function of cardiolipin concentration. Each assay contained 0.5 mg bacteriorhodopsin and the amount of cardiolipin indicated on the abscissa (final volume 5 ml). The upper part shows the proton extrusion by proteoliposomes reconstituted at pH 2.5, the lower part proton uptake by proteoliposomes reconstituted at pH 3.5 (see text and legend to Fig. 1 for further details). It should be noted that purple membrane alone shows negligible light-dependent pH changes.

distinct peaks of radioactivity in the void volume, which coincided with the bacteriorhodopsin peak. Addition of [^{3}H]lactose after formation of proteoliposomes showed no detectable activity in the void volume. The internal volume calculated from the radioactivity in the void volume was about 3 μl/mg lipid for proteoliposomes prepared at pH 2.5 and 6 μl/mg lipid for those prepared at pH 3.5. It remains to be shown that this lactose impermeable space represents the internal volume of proteoliposomes involved in light-dependent proton transport.

The pH dependence of proteoliposome reconstitution was studied using several other lipids. Phospholipids in which the pK of the phosphate group might be expected to be similar to that of cardiolipin (p$K \simeq 3$) showed a similar dependence on the pH during reconstitution. For example, phosphatidylglycerol from *E. coli* gave rise to acidifying vesicles at pH 2.5, while reconstitution at pH 3.5 yielded preparations which catalyzed light-dependent alkalinization. The monomethylester of dimyristoylphosphatidic acid (kindly provided by Dr. H. Träuble), which has a p$K \simeq 3.5$ [13], resulted in acidifying preparations at pH < 3.3 while proton uptake was observed at pH > 3.3. The pH value of this transition is significantly higher than that observed for cardiolipin (cf. Fig. 2). Purified soy bean lecithin (p$K \simeq 1.5$, cf. ref. 13) shows a distinctly different behavior. While this lipid is rather ineffective in reconstitution (compare ref. 7), Fig. 4 shows that acidifying preparations are only obtained at pH 2.5 and a low ratio of phosphatidylcholine to purple membrane. In contrast, cardiolipin gives rise to acidifying preparations at pH 2.5 irrespective of the lipid/purple membrane ratio (cf. Fig. 3).

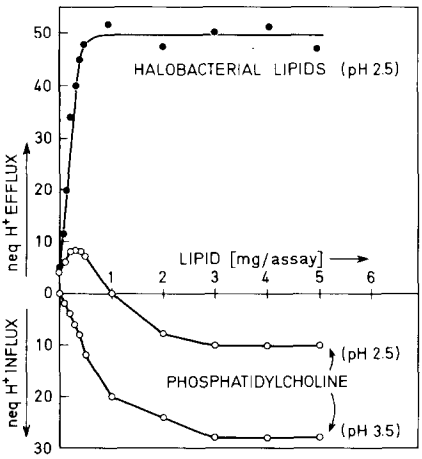

Fig. 4. Extent of proton flux as a function of the concentration of phosphatidylcholine or halobacterial lipids. The experimental protocol was the same as that described in the legend to Fig. 3. Phosphatidylcholine purified from crude soy bean phospholipids (Sigma Chemical Co.) or halobacterial lipids replaced cardiolipin. The pH during reconstitution is indicated.

Of particular interest is the behavior of the lipids present in the purple membrane. Fig. 2b shows that the use of total lipid extracts from *H. halobium* [2] gives rise to acidifying vesicles below pH 3.9 while above this pH alkalinizing preparations are obtained. In agreement with the behavior of cardiolipin, these acidic lipids produce acidifying vesicles at pH 2.5 at all ratios of lipid to protein (cf. Fig. 4). Comparison of Figs. 3 and 4 suggests that the lipids present in the purple membrane may determine the formation of acidifying preparations at a low phosphatidylcholine to protein ratio.

With all samples studied, light-dependent pH changes were readily reversible and highly reproducible in successive light-dark cycles. They were inhibited by uncoupler and the H^+/K^+-antiporter nigericin. The pH changes observed cannot be accounted for by stoichiometric proton release from bacteriorhodopsin. The light intensities used in our experiments produce only negligible steady-state concentrations of deprotonated intermediates of the photochemical cycle (cf. also Fig. 3). Furthermore, proteoliposomes reconstituted with cardiolipin retain lactose, suggesting the presence of closed vesicular structures. These arguments support the contention that the light-dependent decrease in pH is caused by active proton extrusion while the reverse change is caused by active proton uptake. Net proton extrusion indicates a preferential orientation of bacteriorhodopsin similar to that observed in whole cells.

The experiments point to an important role for the surface charge of the lipid vesicles, and possibly for the lipids of the purple membrane in determining the orientation assumed by bacteriorhodopsin in the reconstituted vesicles. The transition from acidifying to alkalinizing proteoliposomes appears to occur in the region of the pK of the lipid phosphate groups. Therefore the formation of acidifying proteoliposomes apparently depends on a partial neutralization of the polar head groups.

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