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THE PREPARATION OF LIPID-DEPLETED BACTERIORHODOPSIN

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Summary

Bacteriorhodopsin, the protein of the purple membrane of *Halobacterium halobium*, was freed to the extent of 90—95% from the natural membrane lipids without loss of function. The residual lipid corresponded to less than 1 mol/mol of bacteriorhodopsin. Delipidation was achieved by treatment of the purple membrane with a mixture of the detergent dimethyldodecylamine oxide and sodium chloride. The detergent was removed by dialysis or by sucrose density gradient centrifugation. Analysis of the lipids removed and those still bound to bacteriorhodopsin was facilitated by the use of purple membrane preparations labelled with ^{35}S, ^{32}P, or ^{14}C. The composition of the residual lipids associated with bacteriorhodopsin was similar to that of the total lipid in the purple membrane.

Introduction

Biochemical studies of the interactions between membrane proteins and phospholipids are clearly desirable for an understanding of the biological membranes. For in vitro studies, a powerful general approach is the reconstitution of defined membrane functions using purified components. If the fatty acyl chains in the phospholipid(s) used in this type of work were to carry activable groups, then there could be the possibility of forming covalent cross links at the points of close contact between the protein surface and the phospholipids. With this aim in view, the preparation of fatty acids and phospholipids with a variety of photosensitive groups has been in progress in this laboratory [1].

For studies on the reconstitution of defined membrane functions, a large number of systems is now available. One such system is the light-driven proton

Abbreviation: DDAO, dimethyldodecylamine oxide; SDS, sodium dodecyl sulfate.

pump constituted by the purple membrane of some extremely halophilic bacteria (e.g. *Halobacterium halobium*) [2—5]. The purple membrane contains a single protein, bacteriorhodopsin, which is complexed with retinaldehyde [2—3].

Pioneering work on the in vitro reconstitution of the proton pump in the purple membrane was carried out by Racker and Stoeckenius [6]. However, purple membrane fragments containing their natural lipid complement were used and the preparation of lipid-free functional bacteriorhodopsin has so far not been accomplished. Because of our interest in studying the protein-phospholipid interaction in the bacteriorhodopsin system, we have now investigated the preparation of lipid-free bacteriorhodopsin, and the present paper describes the results. While a complete lipid-free preparation of functional bacteriorhodopsin has not so far been achieved, 90—95% of the original lipids were removed.

Experimental

Materials

$^{32}P_i$, $^{35}SO_4$ and [1-^{14}C]lauric acid were all purchased from New England Nuclear. [2-^{14}C]Mevalonic acid as a lactone was purchased from Amersham-Searle, and Bio-Gel A-15 m was purchased from BioRad Co. Dimethyldodecylamine was distilled before use. All other materials used were reagent grade.

Methods

Growth of bacteria and isolation of the purple membrane. Halobacterium halbium (strain S-9) was kindly provided by Dr. Stoeckenius. Bacteria were grown in a medium [7,8] for halophilic bacteria, either in 4-l Erlenmeyer flasks containing 2 l each of culture medium, by shaking [9], or in a 15-l cell culture fermentor with an aeration rate of 240 l/h and at a stirring speed of 200 rev./min [10]. The cultures were illuminated by means of a light-bench containing 12 daylight fluorescence lamps (15 W each). The purple membrane was isolated essentially according to Oesterhelt and Stoeckenius [10]. To avoid any proteolytic degradation, the isolation procedure was carried out at 0° C. Purity of the membrane from any contamination with carotenoids present in the red membrane was checked by absorbance characteristics and by sodium dodecyl sulfate (SDS) gel electrophoresis according to the method of Laemmli [11]. The latter technique showed a single band on staining with Coomassie Blue.

Preparation of radioactively labelled purple membrane. Cells (50 ml culture) were grown using 250-ml flasks with side-arms to a density of 50 Klett units. One of the radioactive isotopes (see below) was added and the bacteria were grown to the stationary phase (600 Klett units), at which time the formation of the purple membrane visually appeared to be optimal. Growth medium, which contained 1/3 of the standard amount of yeast extract [7,8], was used for labelling with ^{32}P (5 mCi) (Table I). When ^{35}S (2 mCi) was used, the medium contained $MgCl_2$ instead of $MgSO_4$. For ^{14}C-labelling, a solution of [1-^{14}C]-mevalonic acid lactone (50 μCi) in benzene was evaporated under nitrogen. A 50-ml cell culture grown to 50 Klett units as described above was added and the culture grown to optimal purple membrane synthesis.

Synthesis of the detergent, dimethyldodecylamine oxide (DDAO). The detergent was synthesized by oxidation of dimethyldodecylamine with H_2O_2 as described by Applebury et al. [12]. Synthesis of the ^{14}C-labelled detergent was achieved as suggested by the above authors [12]. [α-^{14}C]lauric acid was converted to the corresponding chloride with thionylchloride and the chloride was reacted with dimethylamine to give the dimethylamide. Reduction with lithium aluminum hydride afforded the tertiary amine, which was treated with H_2O_2 to give the amine oxide.

Removal of the purple membrane lipid: general procedure. The purple membrane (2 mg/ml) was incubated in a solution containing 0.1 M dimethyldodecylamine oxide + 2 M sodium chloride + Tris · HCl, 0.01 M (pH 8.0), for 2 h at 0°C in the dark with stirring. Separation of the bacteriorhodopsin from the released lipid and the detergent was accomplished by one of the following methods:

(a) Gel filtration. The incubation mixture was applied to a column (2.5 × 60 cm) of Bio-Gel A-15m, equilibrated with 0.01 M DDAO + 2 M sodium chloride + 0.01 M Tris · HCl (pH 8.0). The column was eluted in the dark with several bed volumes of the above equilibration buffer.

(b) Sucrose gradient centrifugation. 2 ml of the incubation mixture were layered on top of a linear sucrose gradient (25—40%) which was 0.01 M in DDAO and 0.01 M in Tris · HCl (pH 8.0) and contained a 50% sucrose solution as a cushion. Centrifugation was carried out in a Beckman L3-50 centrifuge using SW27 rotor for 15 h at 25 000 rev./min.

Removal of the detergent. Most of the residual DDAO from the separated bacteriorhodopsin solutions could be removed in one of two ways:

(1) Dialysis. Bacteriorhodopsin solution containing the detergent was dialyzed against 300—400 vols. of 2 M NaCl solution with 2—3 changes every day for 4—5 days. Dialysis was then continued against distilled water or 0.15 M potassium chloride solution for 2 days. The protein, which seemed to undergo aggregation, was then collected by centrifugation at 50 000 × *g* for 0.5 h.

(2) Sucrose gradient centrifugation. This was performed using 30—50% sucrose gradient with a 60% sucrose cushion. Centrifugation was for 15 h at 20 000 rev./min.

Extraction and analysis of lipids. Extraction of total lipids was performed according to Bligh and Dyer [13]. For analysis of lipids remaining in bacteriorhodopsin after DDAO/salt treatment, bacteriorhodopsin solution was first dialyzed against water (4—5 days) to remove the salt and most of the detergent. Residual lipids were then extracted as above.

For separation of the lipids, the two-dimensional thin-layer chromatography system described by Kushwaha et al. [9] was slightly modified as follows: solvent A (first dimension) contained chloroform, methanol and conc. ammonia (50 : 50 : 4, v/v), while solvent B (second dimension) contained chloroform, 80% acetic acid and methanol (60 : 20 : 8, v/v). Precoated silica gel plates (E. Merck) without fluorescence indicator were used. The radioactively labelled lipids were located by autoradiography with Kodak NS-54T film, no screen being used. The spots were scraped out and counted for radioactivity in toluene containing PPO and POPOP (0.32 g and 0.08 g, respectively/100 ml of toluene) in a Beckman scintillation counter (Type, LS-250).

Proton pumping activity of bacteriorhodopsin preparations. This was prepared by the procedure described by Racker and Stoeckenius [6]. The sample was stirred in a test tube surrounded by a water jacket maintained at 20°C. The light source for illumination was a Kodak projector lamp (500 W). The pH changes were measured with a Corning research pH meter, Digital 112, equipped with a combination electrode, Beckman Future No. 39505. The recording of the pH changes was performed by an Esterline Angus Recorder, Speedservo Labgraph W/AZAS.

Results

Preparation of purple membranes with radioactively labelled lipids

Cultures of *H. halobium* were grown in the presence of different radioactive isotopes (see Methods). When [^{14}C]mevalonic lactone was used as a precursor, a 70% uptake of the radioactivity into the cells was observed, and of this, 7.1% was present in the purple membrane. In the case of ^{35}S, an uptake of 5.5% into cells was observed, and 3.5% of the incorporated radioactivity was present in the purple membrane. With ^{32}P, an uptake of 43% was observed into the cells, and of the total incorporation, 1.18% was present in the purple membrane. The purple membrane was isolated as described. No labelling of the protein was detected with any of the radioactive labels as checked by SDS gel electrophoresis (data not shown); however, some [^{14}C]mevalonic acid was incorporated into the retinal moiety of bacteriorhodopsin.

The lipids in the radioactively labelled purple membrane thus prepared were analyzed by autoradiography of thin layer chromatograms of the lipids. The results are shown in Fig. 1.

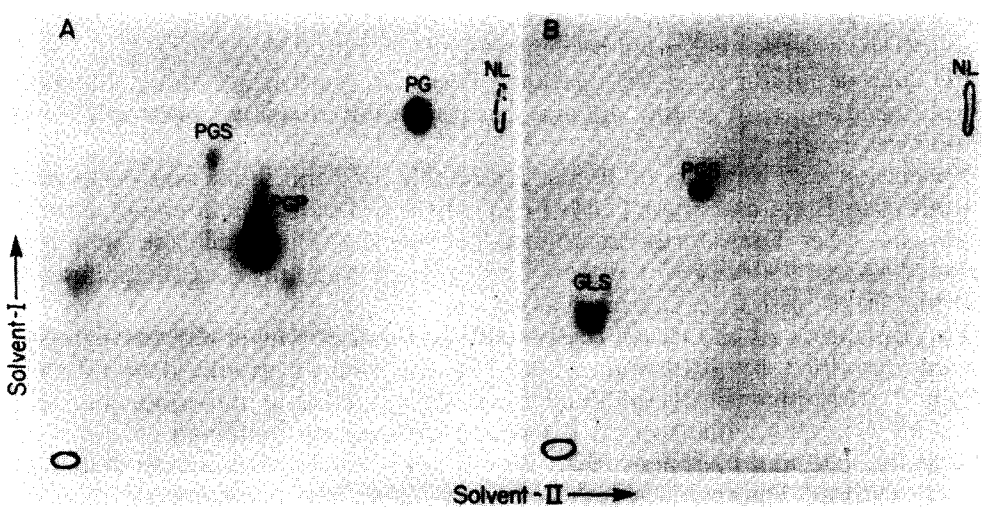

Fig. 1. Autoradiogram of two-dimensional thin layer chromatography on silica gel plates of ^{32}P-labelled (A) and ^{35}S-labelled (B) purple membrane lipids. Solvents I and II are as described in Methods. Identification of different spots is as described by Kushwaha et al. [9]. Abbreviations used are: NL, neutral lipids; GLS, glycolipid sulfate; PGP, phosphatidyl glycerophosphate; PGS, phosphatidyl glycerosulfate; PG, phosphatidyl glycerol.

Removal of lipid by detergent treatment

The purple membrane was incubated with different concentrations of DDAO in the presence of sodium chloride. A minimal concentration of 0.05 M of DDAO was necessary for maximal removal of the lipid. Concentrations between 0.05 M and 0.5 M DDAO gave essentially similar results. Concentrations greater than 0.5 M, while not being any more effective in lipid removal, caused degradation or denaturation of bacteriorhodopsin. A 0.1 M concentration of DDAO was used throughout this work, and since bacteriorhodopsin in DDAO solutions is light-sensitive, all operations were done in semi-darkness.

Separation of the lipids from the protein was achieved by gel filtration on an Agarose column as shown in Fig. 2. To avoid reassociation between the lipids and the protein, a detergent concentration of 0.01 M was maintained during gel filtration. The lipid-depleted bacteriorhodopsin, which was monitored by absorbance at 560 nm and 280 nm, appeared at the void volume. This result indicated the formation of high molecular weight protein aggregates. Any degraded protein, which was included in the gel, was detected by 280 nm absorption.

Incubation of the purple membranes in daylight at room temperature in 0.1 M DDAO + 0.01 M Tris · HCl buffer but without sodium chloride brought about complete removal of the lipid but also resulted in complete loss of the 560 nm absorption, which is characteristic of the functional protein. The chromophore, which results from the specific covalent linkage of the retinal to the protein, could not be restored by the addition of retinal or the purple membrane lipids. Separation of the lipids from the protein was also performed by sucrose density gradient centrifugation as shown in Fig. 3. In the absence of

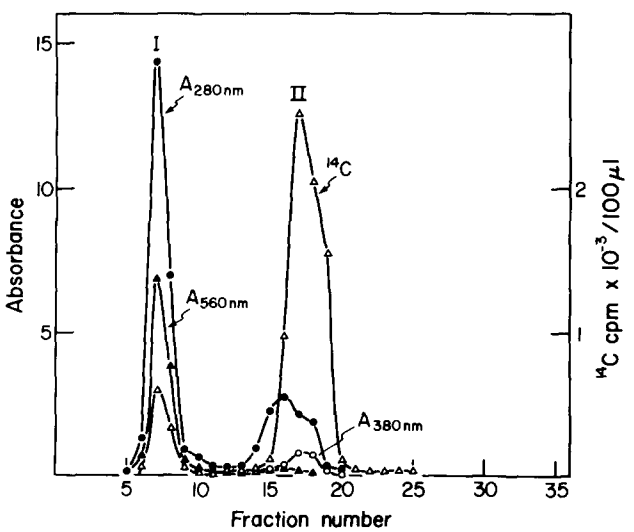

Fig. 2. Separation of the detergent-solubilized lipids from bacteriorhodopsin on a Biogel A-15 m column. ^{14}C-labelled purple membrane (14.2 mg; 2.7 · 10^5 cpm) was incubated in 0.1 M DDAO + 2 M NaCl + 0.01 M Tris · HCl (pH 8) for 2 h at 0°C in the dark. The solution was then applied to a Biogel A-15 m column (2.5 × 60 cm) preequilibrated with 0.01 M DDAO + 2 M NaCl + 0.01 M Tris · HCl (pH 8.0) solution. Elution was with the same buffer, fractions of 4.2 ml each being collected. As judged by the radioactivity in the two peaks, more than 90% of the total lipids were removed from the protein. Results of corresponding experiments using ^{32}P or ^{35}S labels were similar.

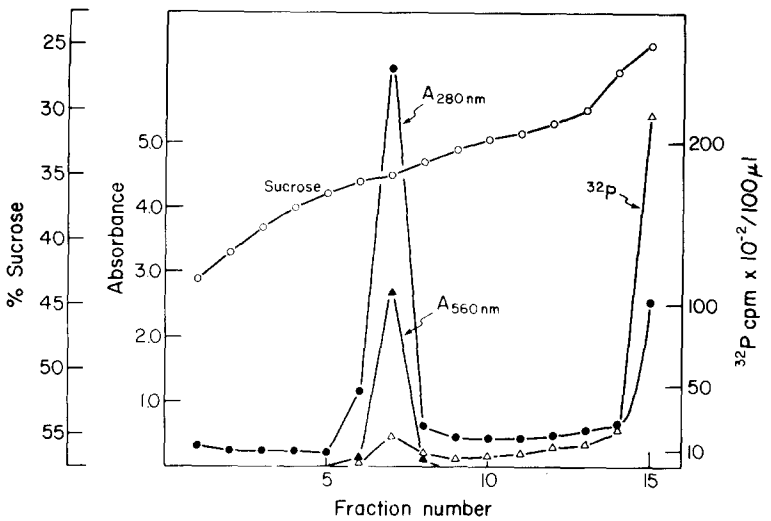

Fig. 3. Separation of bacteriorhodopsin and the solubilized lipids using ^{32}P-labelled purple membrane by sucrose density gradient (25—40% sucrose with a 50% sucrose cushion) centrifugation in the presence of 0.01 M DDAO. ^{32}P-labelled purple membrane (5.6 mg; 1.47 · 10^5 cpm) was incubated in 0.1 M DDAO + 2 M NaCl + 0.01 M Tris · HCl (pH 8) for 2 h at 0°C in the dark, and the solution layered on top of the sucrose gradient. Centrifugation was carried out in a Beckman SW 27 rotor for 15 h at 25 000 rev./min. Fractions of volume 2.4 ml were collected.

the stabilizing effect of sodium chloride, the resulting preparation was more sensitive to degradation in light.

Analysis of the residual lipids

Lipids of the purple membrane have been characterized by Kushwaha and coworkers [9]. Analysis of ^{14}C-labelled residual lipids in the lipid-depleted bacteriorhodopsin showed the presence of all of the characteristic purple membrane lipids (Fig. 4). Quantitative analysis of the residual lipids as well as of the lipids in the total purple membrane was carried out after separation by thin-layer chromatography, and the results are shown in Table I. Firstly, the summation of the radioactivity in the residual lipids and that in the total lipids confirms what is evident in Fig. 2, that the residual lipids in the lipid-depleted bacteriorhodopsin comprise about 5% of the total lipid. The second striking result is that all of the lipids are represented in the residual lipid and that the composition of the residual lipids is very similar to that of the total lipids. An exception seems to be the glycolipid but the content of this lipid is very low (0.2%) and, therefore, the difference is probably not significant. The low value observed for glycolipid is in contrast with the findings of Kushwaha and coworkers [9], who observed that in the purple membrane of *H. cutirubrum*, the triglycosyl diether content was 19.3% of the total lipid. Thus, the lipid composition of the purple membranes from different strains of *Halobacteria* seems to show quantitative differences, although no qualitative difference has been observed [14].

Removal of the detergent

^{14}C-labelled DDAO was synthesized in order to monitor the removal of the

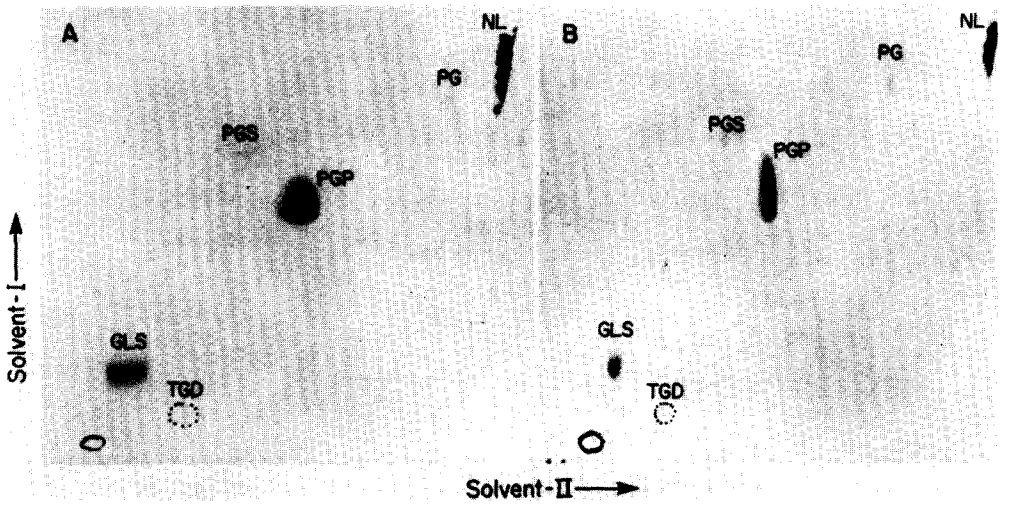

Fig. 4. Autoradiogram of two-dimensional thin layer chromatography on silica gel plates of ^{14}C-labelled purple membrane lipid (A) and residual lipids associated with bacteriorhodopsin after DDAO + NaCl treatment (B). Identification of spots was as described by Kushwaha et al. Abbreviations used are as described in Fig. 1.

TABLE I

ANALYSIS OF THE TOTAL LIPIDS IN THE PURPLE MEMBRANE AND RESIDUAL LIPIDS IN THE LIPID-DEPLETED BACTERIORHODOPSIN

Analysis of the total lipids in the purple membrane and lipids in the lipid-depleted bacteriorhodopsin. In A, ^{14}C-labelled membrane ($2.4 \cdot 10^4$ cpm; 5 mg) was used, the lipids being extracted by the Bligh-Dyer procedure (Fig. 1, 4). For B, the ^{14}C-labelled purple membrane (5 mg; $2.4 \cdot 10^5$ cpm) was treated with DDAO + NaCl as described in the text. The yield as based on A_{560} units was 84%. Residual lipids were extracted as above after removal of the detergent. For quantitative comparison of the residual lipids with the total lipids, the numbers obtained with purple membrane (1/10 specific activity) were multiplied by 8.4 because of the 84% recovery of the membrane containing the residual lipids).

	A. Purple membrane		B. Lipid-depleted bacteriorhodopsin		C. Residual bacteriorhodopsin lipids / Total membrane lipids × 100
	cpm ^{14}C × 8.4	% of total polar lipids	cpm ^{14}C	% of total polar lipids	
Triglycosyldiether	327	0.2	87	1.2	26
Glycolipid sulfate	28 450	18.5	1 625	22.7	5.7
Phosphatidylglycero sulfate	11 415	7.5	129	1.8	1.1
Phosphatidylglycerophosphate	110 947	72.3	5 013	70.2	4.5
Phosphatidylglycerol	2 200	1.4	298	4.1	13.5
Total polar lipids	153 339	100	7 152	100	4.66
Retinal and neutral lipids	22 772 *		11 122 *		

* Bulk of this radioactivity is in retinal. The retinal and neutral lipids which account for about 6% of the total lipids (Kushwaha et al. [9]) were not analyzed further.

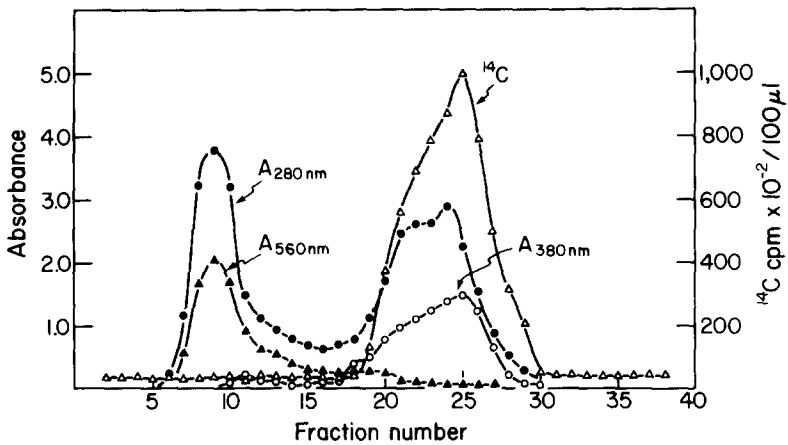

Fig. 5. Preparation of lipid-depleted bacteriorhodopsin using ^{14}C-labelled DDAO and removal of the bulk of detergent on a gel filtration column. The treatment of the purple membrane (15 mg) and separation on a column was as described in Fig. 2, except that DDAO was used in ^{14}C-labelled form (specific activity, 4000 cpm/µmol).

detergent from the protein. Gel filtration of the purple membrane after incubation with ^{14}C-labelled detergent was carried out and the results are shown in Fig. 5. The bulk of the detergent separated nicely from the active protein. Dialysis of the lipid-depleted bacteriorhodopsin containing DDAO against detergent-free buffer afforded a preparation which precipitated as an aggregate containing roughly 2 mol of detergent per mol of the protein (Fig. 6). Centrifugation using a detergent-free sucrose gradient provided an alternative faster method to remove the detergent (Fig. 7).

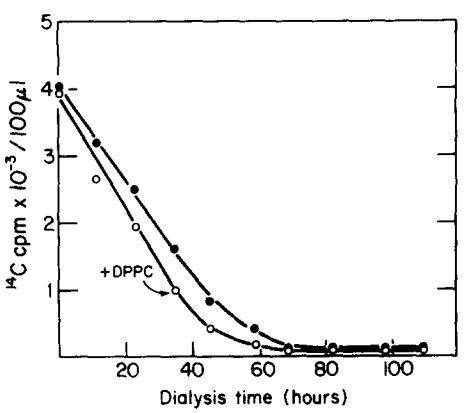

Fig. 6. Removal of the detergent, DDAO, by dialysis. Two solutions of lipid-depleted bacteriorhodopsin (1.4 mg, 1.8 A_{560} units) in 4 ml each of 0.01 M ^{14}C-DDAO (4000 cpm/µmol) + 2 M NaCl + 0.01 Tris · HCl (pH 8) were prepared. To one was added dipalmitoylphosphatidyl choline (DPPC) and the solutions were dialysed against 4 l of 2 M sodium chloride solution with 2 changes every day. Final protein concentration was 13.5 µM, and that of the detergent was about 30 µM.

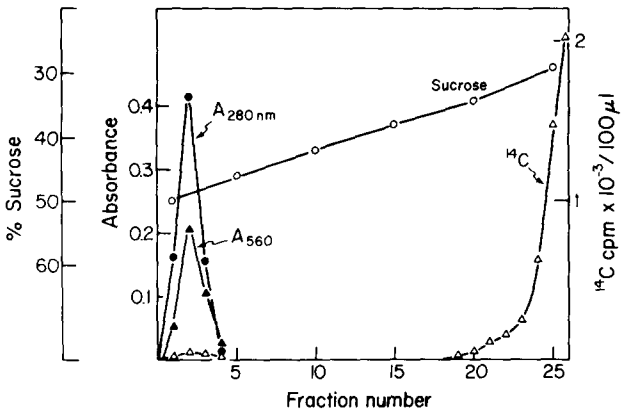

Fig. 7. Removal of the detergent DDAO by sucrose density gradient (30—50%, with a 60% cushion). The lipid-depleted bacteriorhodopsin (0.7 A_{560} units) preparation from Fig. 5 was centrifuged at 25 000 rev./min in a SW27 Beckman rotor for 15 h. Fractions of volume 1.5 ml were collected and analysed for absorbance and radioactivity as shown.

Discussion

In this initial study, a procedure has been described for removal of most of the original lipids present in the purple membrane. Conceptually, the preparation of lipid-free membrane proteins must be achieved by using reagents which compete safely under appropriate conditions with the lipophilic interactions between the natural lipids and the protein. Media containing detergents [15] and organic solvents [16,17] have been most widely used for this purpose. Clearly, the aim of this work was to achieve quantitative removal of the original membrane lipid without loss of the protein function. This has so far not been possible. Bacteriorhodopsin which contains a special set of acidic lipids [9], proved to be very sensitive to gentle treatments with organic solvents and several detergents. The present procedure utilized DDAO, which has been used successfully for solubilization of mammalian rhodopsin [12]. The advantages of DDAO over Triton X-100 or pluronic acids, which were relatively effective, are: (1) it is conveniently removed by dialysis or sucrose gradient centrifugation; (2) it has no absorbance at 280 nm; and (3) it can be prepared readily by synthesis in a chemically pure form. Addition of 2 M sodium chloride was found to be essential for avoiding degradation of the chromophore, which can occur readily in an aqueous solution in the presence of light. It seems likely that sodium chloride stabilized by maintaining delipidated bacteriorhodopsin in an aggregate form. The observations that the lipid-depleted bacteriorhodopsin could be centrifuged down at $100 \cdot 10^3$ g in 1 h and that the preparation eluted from an Agarose 15 m column at void volume indicated that the protein was present in an aggregated form.

Treatments which have so far been found to effect complete removal of the residual lipid also bring about bleaching of the purple retinylidine-protein complex, and attempts to reconstitute the purple color have been unsuccessful. Further, the residual lipid could not be exchanged either by adding an excess of the total purple membrane lipid or by synthetic phospholipids. Assuming the

lipid to protein ratio in the purple membrane to be 10 : 1 (Blaurock, ref. 18), the residual lipid in the present preparation would correspond to less than 1 mol/mol of bacteriorhodopsin. From its tenacious binding, it seems very likely that this residual lipid serves a unique structural role. In the structural model of Henderson and Unwin [19], the residual lipid could occupy the space within each group of three bacteriorhodopsin molecules which form the units in the crystalline arrangement. It is rather surprising, however, that the residual lipid showed no selectivity or specificity in composition in relation to the total purple membrane lipid.

Work on the reconstitution of functional vesicles using the delipidated bacteriorhodopsin and synthetic phospholipids will be reported later.

Note added in proof (Received January 31st, 1977)

A recent report by Happe and Overath [20] has also described experiments on the delipidation of bacteriorhodopsin. Their results are very similar to those recorded here.

Acknowledgements

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