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Isolation and properties of the native chromoprotein halorhodopsin

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The native chromoprotein of the light-driven chloride pump halorhodopsin (HR) was isolated from *Halobacterium halobium* strain L-33 which lacks bacteriorhodopsin but contains 'slow cycling rhodopsin-like pigment' (SR). A membrane fraction was prepared in low salt and dissolved in a high salt medium by the detergents Lubrol PX or octylglucoside. These conditions destroyed the chromophore of SR but not the HR pigment. Chromatography on phenyl-Sepharose and hydroxylapatite produced, in 60% yield, a 230-fold enriched monomeric chromoprotein with an apparent mol. wt. of 20 000. The chromoprotein was stable in 1 M NaCl and 1% octylglucoside and remained stable upon removal of detergent. It reacted with borohydride in the dark and with hydroxylamine in the light. The absorption maximum of the light-adapted state is at 580 + 2 nm and its molar extinction ~50 000/M/cm. Upon illumination in the presence of detergent it was converted into a 410 nm absorbing species with concomitant release of protons. A thermal reconversion to the 580 nm species occurred with a half time of 76 s at −6°C. Blue light absorbed by the photoproduct accelerated the re-conversion as well as the re-uptake of protons. Removal of the detergent prevented the light-induced formation of the 410 nm species. Under these conditions a photochemical behaviour similar to that in intact cells and cell vesicles, i.e., a photocycle in the 10 – 20 ms range was observed. These findings form the basis for functional reconstitution of HR.

Key words: halorhodopsin/native chromoprotein/light-driven ion pump/halobacteria/archaebacteria

Introduction

The family of retinal proteins in Halobacteria now comprises three members. Firstly, bacteriorhodopsin (BR) which mediates phototrophic growth as a photosynthetic pigment (Oesterhelt and Krippahl, 1983). Secondly, halorhodopsin (HR) which acts as a light-driven chloride pump (Schobert and Lanyi, 1982) and is a light-energy transducer but not a photosynthetic pigment because it does not fit the ion specificity of the proton translocating ATPase (Wagner *et al.*, 1981). Thirdly, a pigment occurring in mutant strains which lack BR and HR (and therefore all light-induced ion movements) but still show phototactic activity (Bogomolni and Spudich, 1982). While the light-driven ion pumps combine photochemical cycles of their chromophores with the catalytic cycle of ion translocation in the 10 ms range, the third pigment was called 'slow cycling rhodopsin-like pigment' (SR) because it photochemically reacts to form an intermediate which reconverts only in the second range. On this basis, both HR and SR were shown by flash photolysis to occur in the BR-negative strain L-33 (Bogomolni and Spudich, 1982; Lanyi and Schobert, 1983). Here we report the isolation of the native HR chromoprotein from cell membranes of strain L-33 and describe some properties of the chromoprotein.

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Results

Isolation of the native chromoprotein

HR acts as a light-driven anion pump and, like all other known ion translocating catalysts, can be expected to be an intrinsic membrane protein. The strategy for isolation of the native chromoprotein from L-33 cells, therefore, involved a two-step purification. Firstly, a cell membrane fraction was prepared and as many contaminants as possible removed from the membrane without solubilizing the chromoprotein. Secondly, the membrane was solubilized by detergents in the presence of high salt for chromatographic purification. Table I summarizes the individual steps which led, with 60% yield, to a 230-fold enriched chromoprotein which migrated on SDS-polyacrylamide gradient gels as a single band with an apparent mol. wt. of ~20 000 (Figure 1).

The essential steps in the first half of the purification procedure were preparation of a lysate by dialysis of cell suspensions against water and the preparation of a cell membrane fraction by high speed centrifugation. Membrane subfractionation on sucrose density gradients did not specifically enrich the chromoprotein in any of the fractions, in contrast to BR which predominantly occurs in the purple membrane subfraction. Therefore, the total cell membrane fraction was extracted with Tween 20, a hydrophilic detergent. The yield of chromoprotein was determined by difference spectroscopy of hydroxylamine-treated, bleached samples against an unbleached reference (see 'Properties'). Negligible losses occurred during preparation of the total membrane fraction, confirming that all HR is membrane-bound. Tween 20 extraction at a detergent:protein ratio of 20:1 removed 56% of contaminating protein, mainly species >50 000 dalton apparent mol. wt. (see Figure 1).

The essential steps in the second half of the purification procedure were chromatography on phenyl-Sepharose and hydroxylapatite after solubilization of the membrane. Attempts to solubilize the membrane in the presence of low salt concentrations with ~20 different detergents failed because solubilization was always accompanied by denaturation (decolorization). However, two detergents were found which solubilized the membrane with minimal loss of color in the presence of sodium chloride at concentrations >2 M. Lubrol PX was favoured over octylglucoside in large scale preparations of the chromoprotein for economic reasons although octylglucoside stabilized the native structure more efficiently and therefore was used in the final steps.

Even under the optimal conditions of 1% octylglucoside or 1% Lubrol PX in 4 M sodium chloride, only 50% of the purple color was preserved after solubilization (Table I). Since L-33 cells contain HR and SR, the loss of purple color could be due to either partial decolorization of both chromo-

Table I. Purification of the native chromoprotein HR

Stage of purification	Volume (ml)	Protein (mg)	Pigment (nmol)		Specific pigment content (nmol/mg)	Purification factor	Yield %
			NH_2OH + hν	abs.			
Lysate	400	3810	660		0.17	1	100
Total membrane fraction	61	436	628		1.4	8	95
Tween-washed membrane	13.7	193	518		2.7	16	78
Phenyl-Sepharose chromatography	172	18		253	14	82	39
Hydroxylapatite chromatography	179	8		251	31	182	38
Detergent exchange (phenyl-Sepharose)	27	6		203	34	200	31
Concentration step (Amicon YM 10)	7.6	5		195	39	230	30

The value for a purification from 40 l cell culture is given. Pigment concentration was either determined by difference spectroscopy after hydroxylamine bleaching (NH_2OH + hν) or difference spectroscopy (abs.). An average molar extinction coefficient of 50 000/M/cm was assumed. The final yield of HR is 60% because the 50% loss of purple color upon solubilization is due to SR, not HR.

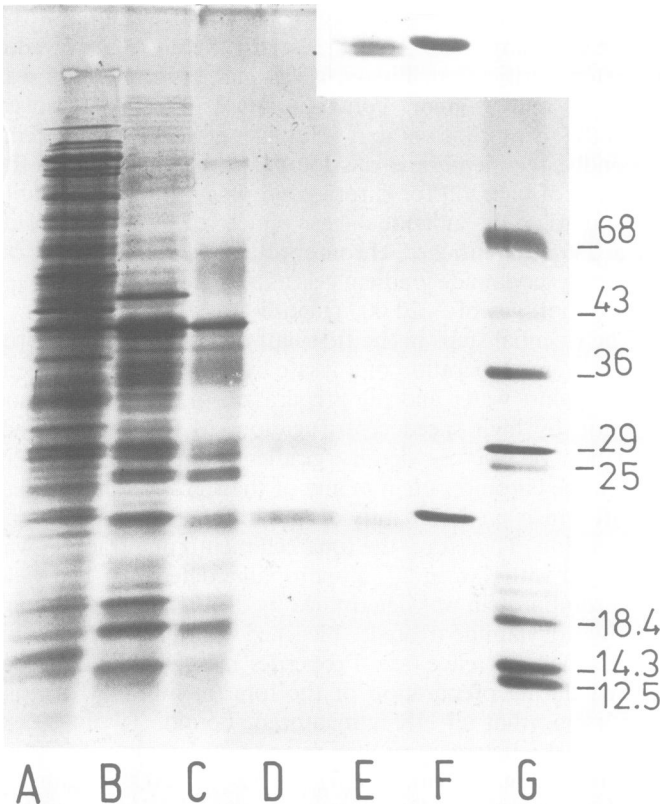

Fig. 1. Analytical SDS-PAGE of the HR chromoprotein-containing fractions from various stages of purification. The gel was fixed with 10% trichloroacetic acid and stained with Coomassie R 250. BR and HR migrate anomalously on the SDS-gel, see text and Lanyi and Oesterhelt (1982). **Lane A**: cell lysate; **lane B**: total membrane fraction; **lane C**: Tween-washed membrane fraction; **lane D**: pooled fractions 10–28 after phenyl-Sepharose chromatography; **lane E**: purified HR chromoprotein; **lane F**: BR; **lane G**: marker proteins with their mol. wts. as indicated on the right side. The inset upper right shows on the left the doublet band of the HR chromoprotein and on the right BR from another SDS-gel with higher resolution.

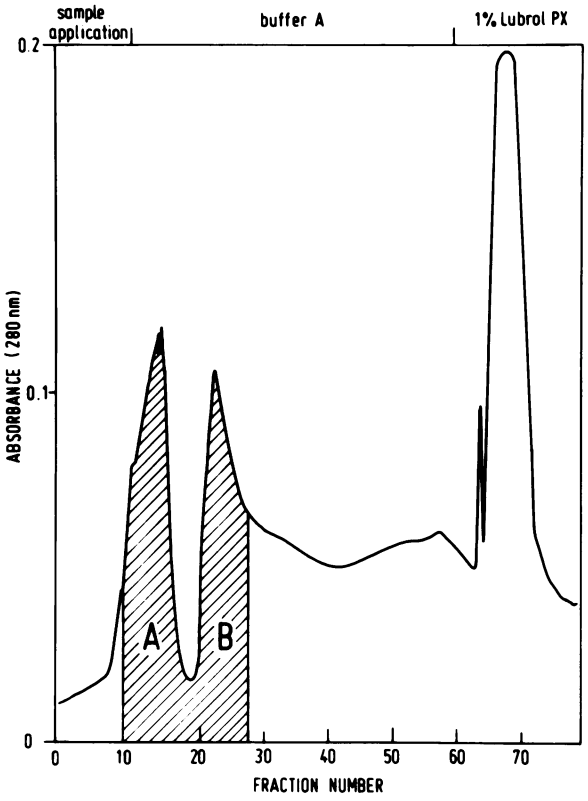

Fig. 2. Hydrophobic chromatography of the Tween-washed membrane solubilized in 1% Lubrol PX/4 M sodium chloride/10 mM MOPS pH 7.0/0.05% sodium azide (buffer A) on phenyl-Sepharose CL-4B. The HR chromoprotein was not retained and eluted in two fractions A and B (shaded area). Washing with 1% Lubrol PX removed the other membrane proteins from the column, especially cytochromes. The operations are indicated on top of the figure.

proteins or selective decolorisation of one of them. We interpreted the loss in color as selective denaturation of SR for the following reasons. Firstly, we prepared a Tween-washed membrane from F1x3 cells which contain only SR (i.e., HR and BR are absent, Bogomolni and Spudich, 1982). Upon solubilization, the purple color completely disappeared. Secondly, it is known that, in contrast to BR and HR, our conditions of solubilization destroy the photochemical activity of SR (Bogomolni and Spudich, 1982). Thirdly, the observed 50% loss of color is consistent with the flash photolysis data of Schobert and Lanyi (1982) and our own preparations (Tittor, unpublished data) which describe a HR:SR ratio of ~1:1 in strain L-33.

After solubilization of the Tween-washed membrane, only minor losses of color occurred during the purification of the HR chromoprotein. Chromatography on a phenyl-Sepharose column removed large amounts of contaminating proteins, in particular all cytochromes, which remained bound while the chromoprotein eluted in two fractions A and B (Figure 2). We assume that the separation into fractions A and B is a

chromatographic artifact because chromatography at 4°C in the dark produced only one fraction and the material from fractions A and B when purified to homogeneity showed the same protein band on SDS-PAGE. Furthermore, the same two fractions were also observed when BR, which is definitely a single protein species, was prepared from S9 cells in exactly the same way as HR from L-33 cells.

The purple-colored eluate from the phenyl-Sepharose column still contained material with a broad absorption band at ~400 nm. Hydroxylapatite chromatography separated the HR chromoprotein eluting in the void volume from the yellow material which only eluted in the presence of 100 mM sodium phosphate (compare the dark spectra of Figure 5A and B). Difference spectroscopy of dithionite-reduced samples *versus* oxidized samples demonstrated the absence of cytochromes in this material and chemical analysis proved retinal to be responsible for the yellow color. Therefore, we assumed that denatured SR and/or denatured HR chromoprotein were contaminating the preparation. This assumption was confirmed by preparation of a Tween-washed membrane from Flx3 cells. As mentioned already, upon solubilization the purple color disappeared and upon phenyl-Sepharose chromatography the material absorbing at ~400 nm eluted in the fractions corresponding to A and B in a HR chromoprotein preparation.

The last step in the purification of HR chromoprotein was an exchange of Lubrol PX for octylglucoside on a second phenyl-Sepharose column and the concentration of the chromoprotein by ultrafiltration in an Amicon cell with a hydrophilic YM 10 filter. Figure 1 demonstrates the efficiency of the individual purification steps and the purity of the final product. SDS-PAGE of the final product for a longer period of time resolved the chromoprotein into two bands (Figure 1, inset upper right). The significance of this doublet in relation to the question of purity and mol. wt. of SR will be discussed below. The chromoprotein prepared as described could be stored at 4°C in the dark without loss of color for several weeks.

Properties of the HR chromoprotein

The spectrum of the purified HR chromoprotein in 1 M salt and 1% octylglucoside is shown in Figure 3. The absorption maximum was at 580 ± 2 nm and was blue-shifted compared with the absorption maximum of 588 nm in cell vesicles (Lanyi and Weber, 1980). BR under the same conditions had an absorption maximum of 565 nm (light-adapted) or 555 nm (dark-adapted) compared with 570 nm (light-adapted) before addition of salt and octylglucoside. The broad featureless absorption band of HR had the same half width of 124 nm as BR examined under identical conditions. In liquid nitrogen the half width became smaller (86 nm) and the absorption band asymmetrical in shape indicating two shoulders at 548 and 600 nm.

The extinction coefficient of the HR chromophore was determined with BR of the purple membrane as a reference (ϵ_M = 63 000/mol/cm) in the following way. Purple membranes which were isolated from wild-type Halobacteria, strain S9, and solubilized in 1 M salt and 1% octylglucoside did not change the absorbance and allowed the reaction of both chromoproteins with hydroxylamine in light under identical conditions (Figure 5C). The decrease in absorption at the maximum of chromophore absorption and the increase in absorption at the maximum of retinaloxime absorption were measured in both samples. With the assumption that the

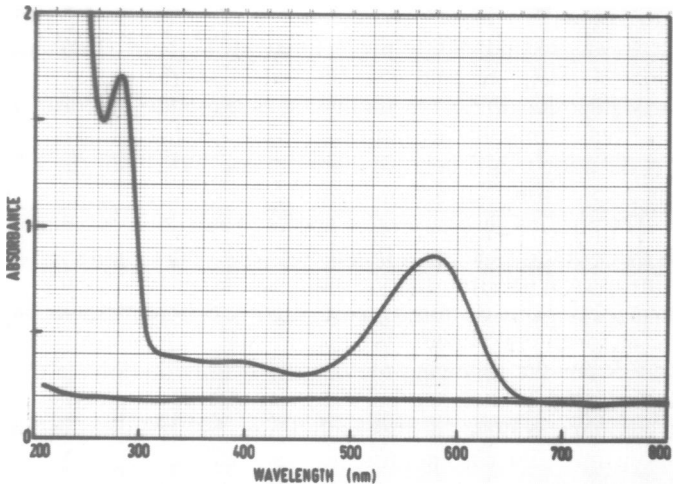

Fig. 3. Absorption spectrum of the purified HR chromoprotein in 1% octylglucoside/1 M sodium chloride/10 mM MOPS pH 7.0/0.05% sodium azide.

retinaloximes, formed in both samples, occurred in the same ratio of *cis-trans* and *syn-anti* isomers, we calculated the extinction coefficient of HR chromophore. As a result, a molar extinction of HR of ~50 000 was obtained, which is close to the value reported for HR in cell vesicles (Lanyi and Weber, 1980).

As in the spectrum of BR or visual pigments, a weak absorption band, called the β-peak, was observed in the HR spectrum. However, this absorption could be due to traces of decolorized HR and not to intrinsic absorption of the native chromophore structure (see below). The 580:280 ratio of absorption for the HR chromoprotein was 1:1.85 and nearly equal to that found for BR (1:1.7 under the same conditions). From these ratios, the extinction coefficients and the known tryptophan content of BR it could be estimated that the retinal:tryptophan ratio is similar in both proteins, but that HR contains one tryptophan residue less than BR.

Although the HR chromoprotein was relatively stable it slowly lost its chromophore, especially if permanently exposed to light. The product was the yellow coloured material absorbing at ~400 nm with the maximum at 392 nm (Figure 5B) which did not reconvert thermally or photochemically to the native chromoprotein. BR under the same conditions was much more labile and could not be isolated from wild-type cells by the same procedure without considerable amounts of decolorized protein contaminating the preparation. For HR, immediate decolorization also occurred if salt was removed, but the chromoprotein remained stable in the presence of salt if octylglucoside was dialysed away. Under these conditions no visible aggregation of the protein occurred. This is a surprising result for an intrinsic membrane protein but may be explained by residual detergent molecules of Tween, Lubrol PX and octylglucoside which had not been removed from the protein by dialysis.

The apparent mol. wt. of HR chromoprotein was 20 000 on SDS-gels, i.e., ~1000 daltons less than the apparent mol. wt. of BR (21 000) which, however, has a true mol. wt. of 26 000. Based on the assumption that HR migrates atypically on SDS-gels, as does BR, a true mol. wt. of 25 000 for HR chromoprotein had been suggested (Lanyi and Oesterhelt, 1982). The mol. wt. of the native HR chromoprotein was also compared with BR by gel filtration in high salt and detergent. Both proteins were chromatographed under identical condi-

tions on a TSK-G 3000 SW column. Neither of the two proteins was excluded but they eluted at 17.5 ml (BR) and 18.5 ml (HR), respectively. BR, when solubilized by 1% octylglucoside with or without salt, is a monomeric protein (H.Michel, unpublished observation). From the result of the gel filtration experiment we therefore concluded that the HR chromoprotein is also monomeric under these conditions.

The purified chromoprotein appeared as a doublet band on high resolution SDS-gels (Figure 1, inset upper right). Two-dimensional gel electrophoresis did not reveal additional protein species but demonstrated a slightly different isoelectric point for the two bands. We assume that both bands belong to HR for the following reasons. (i) If only one of the two bands contained retinal but both proteins have an average tryptophan content, a drastically lower 580:280 ratio in the spectrum of the chromoprotein would be expected. (ii) When HR activity was induced in retinal-negative cells (strain L-07) by addition of retinal, both bands increased as seen by Coomassie stain density on SDS-gels (P.Hegemann, unpublished observation). (iii) Amino acid analysis, cyanogen bromide cleavage and sequence analysis of this material led to the conclusion that the two protein bands must be derived from the same polypeptide chain (Hegemann and Oesterhelt, in preparation).

The isolated HR chromoprotein could be reduced with borohydride in the dark to a fluorescent retinylprotein (RP, see below) but reacted with hydroxylamine only in light (Figure 5C). Dark reduction of HR chromoprotein by borohydride at the stage of the Tween-washed membrane is shown in Figure 4. As a reference, RP from BR which had been prepared by illumination of purple membranes in the presence of borohydride (Schreckenbach *et al.*, 1977) was either applied separately or mixed with the Tween-washed and reduced membrane. As is evident from the pattern of fluorescent bands in Figure 4, only one RP positioned just below RP from BR was formed. When the same experiment was repeated with a Tween-washed membrane from Flx3 cells, no fluorescent band could be detected (not shown). Thus, SR was not reduced to a RP by borohydride in the dark to an extent comparable with HR.

In intact cells the chromoproteins of HR and BR have different absorption maxima (BR: 570 nm, light-adapted; HR: 588 nm) and photochemical cycles of approximately the same frequency (~ 100/s) but involving different intermediates. In the isolated state the chromoprotein of HR absorbed at 580 nm (HR_{580}) compared with 565 nm for BR and Figure 5 shows that illumination at $-6°C$ produced a photoproduct absorbing at 410 nm (HR_{410}) which reconverted with first order kinetics ($>95\%$) with a half time of 76 s into the HR_{580} form. BR, under the same conditions, reconverted to its original state in less than the time resolution of the instrument (300 ms). Figure 5A and B compare the photochemical reaction of the HR chromoprotein at two different states of purity, before and after removal of the 400 nm absorbing material by hydroxylapatite. No difference in half times of regeneration was observed between the two samples. Blue light accelerated the regeneration of HR_{580} (Figure 5D), but not fully to 100%. Part of the reconversion (the last 10%) occurred independent of blue light illumination. It is interesting to note that this 'blue light acceleration' is also observed in BR (Oesterhelt *et al.*, 1975; Hess and Kuschmitz, 1977; Danchazy *et al.*, 1978).

The formation of the 410 nm product was accompanied by a reversible release and uptake of protons. Figure 6 shows these light-induced pH changes which account for a stoichiometry of ~ 1 proton per chromophore. More detailed studies will have to be carried out to establish whether this proton release and uptake is due to a Schiff's base deprotonation-protonation reaction in HR.

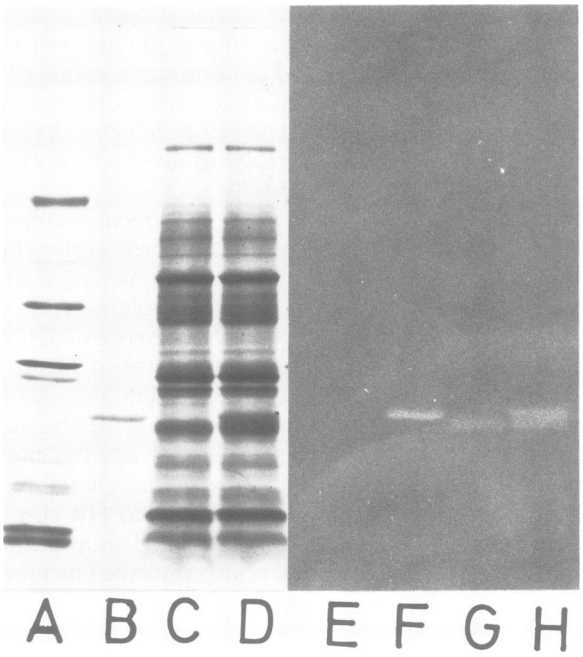

Fig. 4. Comparison between the RPs from BR and HR by analytical SDS-PAGE. **Lanes A–D**: Coomassie stain of marker proteins **(A)**, RP from BR **(B)**, sodium borohydride-reduced Tween-washed membrane from L-33 cells **(C)**, and an equal mixture of **B** and **C (D)**; **lanes E–H**: fluorescence pattern of the same SDS-gel before staining with Coomassie R 250.

The most important property of the isolated HR chromoprotein is the fact that removal of octylglucoside does not lead to a loss of color but also restores the photochemical activity of the protein virtually to its behaviour in the membrane-bound state. Reconversion to HR_{580} occurs with a half time of $\sim 15-20$ ms, which is a little slower than the 10 ms photocycle in cell vesicles. This difference might be due to the presence of small residual amounts of detergent and the lack of lipids in the preparation. However, this experiment clearly shows that reconstitution of HR into liposomal systems by dilution of the octylglucoside-solubilized protein should be possible and that no formation of the 410 nm product will interfere with measurements of the photocycle or chloride transport upon continuous illumination.

Discussion

HR is the first known light-driven anion pump. At present it is not known whether the pump comprises several protein components or whether it is just the chromoprotein we have isolated. To prove this it will be necessary to reconstitute the function by incorporation of the chromoprotein into liposomal systems or black lipid membranes. Important prerequisites for this are two properties of the isolated chromoprotein. First, the detergent octylglucoside can be removed without denaturation of the protein and second, upon removal of the detergent, a photocycle as observed in intact cells is re-established in the molecule. The Tween-washed membrane turned out to be a very suitable material for

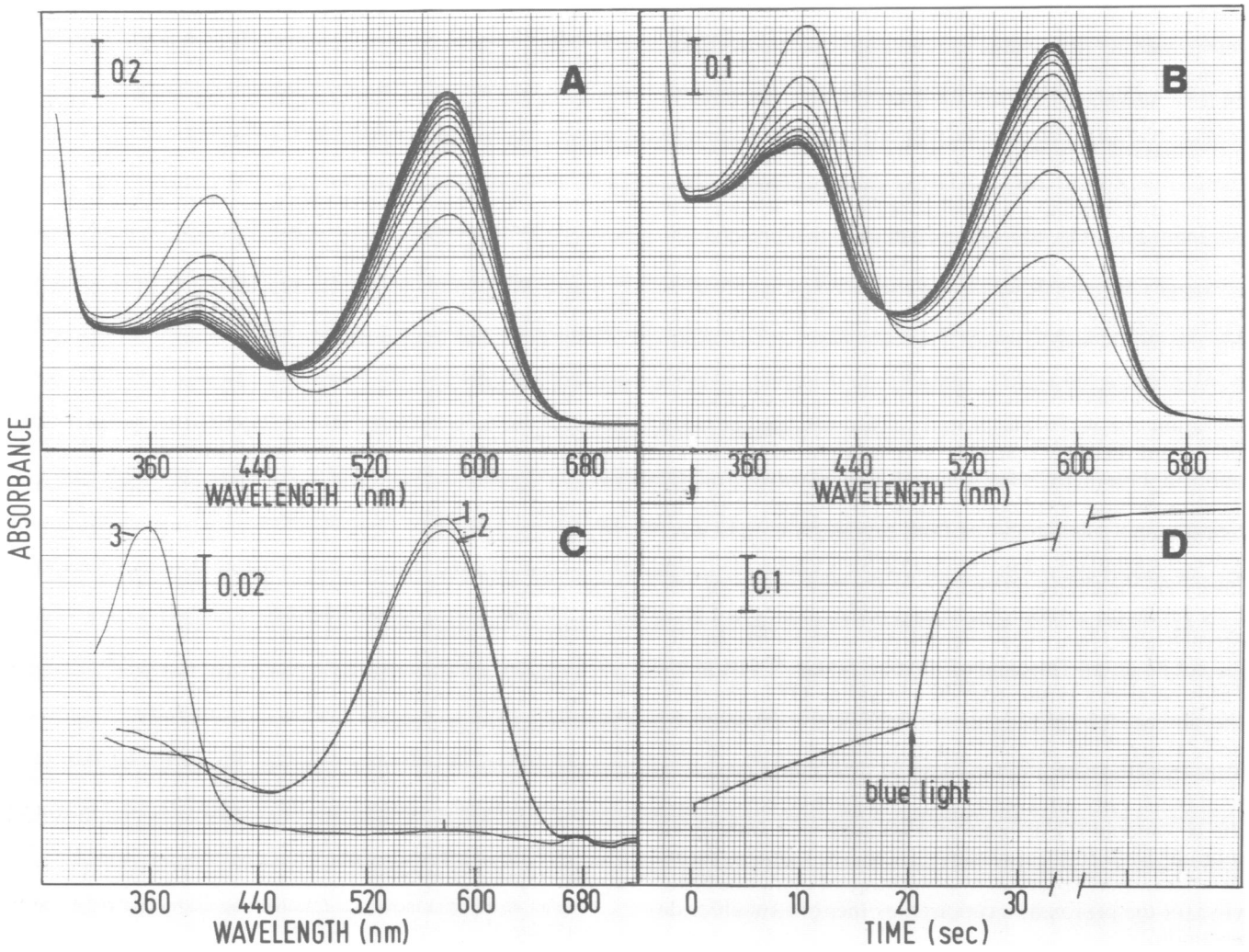

Fig. 5. The photoreaction of the HR chromoprotein **(A)** after and **(B)** before hydroxylapatite chromatography in 1% octylglucoside/4 M sodium chloride at −6°C. The samples were illuminated (OG 515 filter) for 1 min and the spectra recorded continuously after turning off the light. The first spectrum has the highest absorption at 410 nm and the lowest at 580 nm. Time difference between the spectra was 1 min. The halft time was evaluated from the kinetic experiment at constant wave length. **(C)** Reaction of the HR chromoprotein with hydroxylamine (0.2 M, pH 7.0) in light (OG 515 filter). **Curve 1**: HR chromoprotein before bleaching; **curve 2**: after addition of hydroxylamine in the dark; **curve 3**: after 15 min illumination (complete bleach). **(D)** Blue light (390−415 nm interference filter) acceleration of the regeneration of HR_{580}.

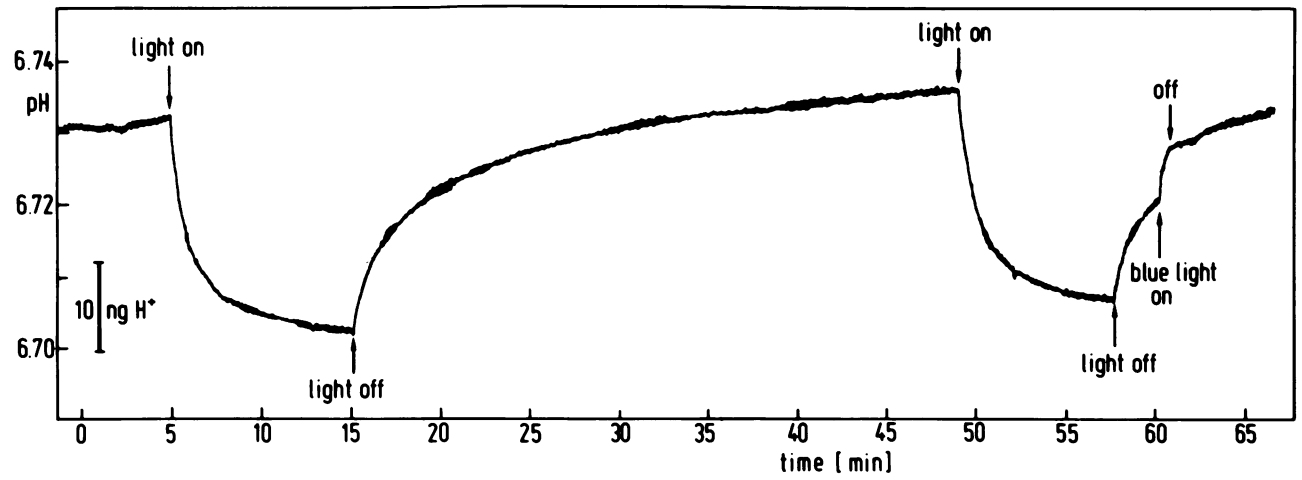

Fig. 6. Reversible proton release and re-uptake of the HR chromoprotein upon illumination. The pH changes during intermediate formation of HR_{580} were measured in a thermostatted cuvette illuminated by light from a 150 W projector (OG 515 filter, see Hess and Oesterhelt, 1973). Regeneration of HR_{580} was accelerated by illumination with light filtered as in Figure 5D.

photophysical studies on the ion specificity of the photochemical cycle in HR but also allowed us to validate the black lipid membrane method for functional reconstitution. The membrane adsorbed to black lipid membranes has already allowed demonstration of chloride-dependent light-induced electric responses (Hegemann *et al.*, in preparation).

The comparison of BR and HR revealed differences and some surprising similarities. During purification BR was less stable than HR. On the other hand, in the presence of detergent, the photocycle of BR remained unchanged in the 10 ms range whereas HR was slowed down considerably in its thermal regeneration. For BR, such a retarded regeneration during the photochemical cycle has been observed under different conditions, e.g., presence of high salt and ether (the so-called 412 nm intermediate in BR). In both cases of retarded regeneration the reversible proton release and uptake were observed and the back reactions were accelerated by the absorption of blue light. This indicates a similarity in retinal protein interaction and detailed studies on a comparison of the chromophoric structures of both proteins are underway. Whereas the retarded regeneration provided the basis for the discovery of the first intermediate in the photocycle of BR and the reversible proton uptake and release connected to it (Oesterhelt and Hess, 1973) the 410 nm intermediate in HR might not be an intermediate of the photocycle of HR in intact cells because it is also formed at alkaline pH in the dark in the absence of chloride or by light in the presence of chloride (Lanyi and Schobert, 1983). However, it has not been identified so far as an intermediate of the photochemical cycle in intact cells. In any case, the analysis and comparison of the chromophores of both proteins and their changes in light are relevant to the question of how two retinal proteins drive ion transport processes by absorption of light, one being specific for the proton as a cation, the other one for chloride as an anion.

For the purification of HR it was fortunate that, under the conditions used, SR was denatured with respect to its chromophore. Therefore, the analysis presented in this paper concerned exclusively the HR chromophore, although it is difficult to exclude that SR apoprotein might contaminate our HR preparation. In a recent report the apoprotein of SR was considered to have a mol. wt. of 25 000 (Spudich *et al.*, 1983). We believe the experiments reported here, together with our unpublished experiments, exclude the possibility that SR is a contaminant of our HR preparation because our experimental data show that SR does not have a mol. wt. of 25 000. Another protein which has a similar mol. wt. and could contaminate our preparation is a 26 000 glycoprotein which was described by Wieland *et al.* (1980). It migrates on SDS-gels slightly slower than BR (Sumper, personal communication), is removed by hydroxylapatite chromatography and, in addition, occurs in very small amounts in the cell. We further believe that the doublet band appearance of HR is due to a modification of the polypeptide chain because, as yet, we have been unable to detect sequence heterogeneity. The same multiple band appearance has been found for other proteins involved in chemotaxis (Hazelbauer and Engstroem, 1981) and for BR (Steiner and Oesterhelt, in preparation).

Materials and methods

Materials

Phenyl-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and hydroxylapatite (Biogel HT) from Bio-Rad (Richmond, VA). Tween 20 (polyoxyethylenglycol-sorbitolestermonolaureate), Lubrol PX (polyoxyethylenglycol-(9,10)-laurylalcohol) and octylglucoside(n-octyl-β-D-glucopyranoside), were purchased from Sigma (Taufkirchen, FRG), DNase I (bovine pancreas, grade II from Boehringer, Mannhein, FRG) and MOPS (3-morpholinopropane-sulfonic acid) from Serva (Heidelberg, FRG). All other chemicals and reagents were of analytical grade and obtained from Merck (Darmstadt, FRG).

Solutions

Buffer A = 1% Lubrol PX, 4 M sodium chloride, 10 mM MOPS pH 7.0, 0.05% sodium azide; buffer B = 4 M sodium chloride, 10 mM MOPS pH 7.0, 0.05% sodium azide; buffer C = 1% octylglucoside, 1 M sodium chloride, 10 mM MOPS pH 7.0, 0.05% sodium azide; buffer D = 10 mM MOPS pH 7.0, 0.05% sodium azide. For all solutions, deionized and distilled water was used. Buffers of high ionic strength (e.g., >1 M salt) were filtered through Millipore filters (0.45 μm).

Isolation of the chromoprotein

Halobacterium halobium strains S9, L-33, and Flx3 (obtained from J.Spudich) were grown as described previously (Hegemann *et al.*, 1982). Purple membranes were isolated according to Oesterhelt and Stoeckenius (1974) and reduced with borohydride as described by Schreckenbach *et al.* (1977). The preparation of membrane fractions or the chromoproteins BR and HR followed the procedure given below for all strains.

Preparation of a lysate

If not otherwise mentioned, all operations were carried out at room temperature and in dim light. Cells from a fresh 40 l culture were centrifuged at 3000 *g* for 60 min in a centrifuge (Stock, Marburg, FRG) yielding a cell mass of ~120 *g* wet weight. Cells were resuspended in 400 ml basal salt pH 7.0 (medium without peptone), centrifuged for 15 min at 14 000 *g*, resuspended in 250 ml basal salt containing 5 mg of DNase I and dialysed overnight against 10 l of water at 4°C.

Total membrane fraction

The lysate (~400 ml) was centrifuged at 10°C for 1 h at 200 000 *g* and the pellet resuspended by homogenization in a Potter Elvejhem homogenizer with 200 ml 0.1 M sodium chloride and pelleted again. Then it was resuspended in 60 ml of 0.1 M sodium chloride and the protein concentration adjusted to ~7 mg/ml.

Tween-washed membrane

60 ml of the total membrane fraction were mixed with 180 ml buffer D containing 5% Tween 20 (protein:detergent ratio 1:20). Aliquots of 24 ml were added to tubes of a Kontron TST28 swingout rotor and underlayed successively with 6 ml buffer D containing 25% sucrose (w/w) and 3 ml buffer D containing 45% sucrose (w/w). After centrifugation at 80 000 *g* for 14 h at 10°C, the deep red material in the lower part of the 25% and the upper part of the 45% sucrose layer was collected from the tubes, combined and diluted with 0.1 M sodium chloride to a final volume of 250 ml. After centrifugation at 10°C for 1 h at 200 000 *g* the pellet was washed with 100 ml 0.1 M sodium chloride and resuspended finally in 13 ml of the same solution. The protein concentration was ~15 mg/ml.

Phenyl-Sepharose chromatography

10 ml of the Tween-washed membrane were mixed with ~120 ml of buffer A to assure a protein to Lubrol PX ratio of 1:8. After stirring the mixture for 30 min at room temperature in the dark, centrifugation for 30 min at 200 000 *g* at 10°C separated a red colored supernatant from a yellow-white pellet. The supernatant was applied to the top of a column filled with phenyl-Sepharose (bed volume 5 cm x 5 cm) and equilibrated with buffer A. The purple colored material was not significantly retained by the phenyl-Sepharose upon elution with buffer A but separated into two fractions, A and B.

Hydroxylapatite chromatography

Fractions A and B were combined (170 ml) and applied to a hydroxylapatite column (1.6 cm x 2 cm) which had been washed with 20 bed volumes of water and then with the same volume of buffer A. The purple colored material was not adsorbed and eluted with the voild volume. Yellow material was eluted with buffer A containing 0.1 M sodium phosphate.

Detergent exchange

The purple colored fractions were combined and applied to a second phenyl-Sepharose column (2.6 cm x 10 cm) which has been equilibrated with the detergent-free buffer B. The column was washed with 3 volumes of buffer B and then with 50 ml of buffer C. The chromoprotein eluted as a sharp band and was further concentrated in an Amicon cell (YM 10 filter).

Regeneration of phenyl-Sepharose

Regeneration of the phenyl-Sepharose was essential for repeated use. The

material was first washed with three bed volumes 1% Lubrol PX and the same volume of water, 50% ethanol and again water before emptying the column. After degassing, the column was repacked and equilibrated with ~600 ml of buffer A.

Gel filtration

A TSK-G 3000 SW column (LKB Ultro Pac, 7.5 mm x 600 mm) connected to a Gynkothek model 600/200 high pressure pump was used. The column was equilibrated and eluted with buffer B containing 1% octylglucoside with a rate of 6 ml/h at a maximum pressure of 30 bar. 200 μl of a chromoprotein solution were applied.

Spectroscopic assays for chromoproteins

The sample (200 μl) was mixed with 1.8 ml buffer D containing 1% Lubrol PX and 200 μl 2 M hydroxylamine pH 7.0 was added. One half of the sample was illuminated with a 900 W Xenon lamp, filtered through heat filters and 1 mm of an OG 515 (Schott) filter for 15 min (85 mW/cm²). The other half of the sample was kept in the dark. The difference spectrum was recorded with an Aminco DW2 spectrophotometer and an extinction coefficient of 50 000/M/cm used for calculation of pigment concentration (Lanyi and Weber, 1980). Alternatively, the amount of chromoprotein was estimated from the decrease in absorbance at 570 nm in the sample after addition of SDS (Ogurusu *et al.*, 1982).

Borohydride reduction of chromoproteins

2.5 ml of the Tween-washed membrane (15 mg/ml) were mixed with 2.5 ml of 2% sodium borohydride solution and stirred for 10 min at 4°C in the dark. The sample was washed three times with 10 ml of water by centrifugation for 30 min at 200 000 *g* at 4°C and finally resuspended in 500 μl of water.

Analytical procedures

Protein fractions containing the chromoprotein or the pure protein were analyzed on gradient (9–24%) SDS-PAGE according to Laemmli (1970). Two-dimensional electrophoresis was carried out according to O'Farrell (1975) and Pedersen *et al.* (1976). Protein was determined according to Warburg and Christian (1942) which is a convenient method and applicable to membrane fractions as well as to detergent-solubilized samples but always gave values higher by a factor of 4 than protein determination according to Lowry with same samples. This Warburg and Christian method, however, could only be applied to membrane fractions because detergent solubilization introduced erroneous results. The values in Table I were therefore measured by the method of Warburg and Christian and divided by 4. Octylglucoside was analyzed by the phenol sulfuric acid reaction for carbohydrates (Ashwell, 1966) and sodium chloride by conductometry. The reversible bleaching of the chromoproteins was carried out in an Aminco DW2 spectrophotometer and the actinic light projected onto the sample by a light guide. Proton release upon formation of an intermediate was measured exactly as described by Oesterhelt and Hess (1973).

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