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Isolation and characterization of the retinal-binding component of halorhodopsin

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Halorhodopsin (HR) was reconstituted in cell vesicles prepared from *Halobacterium halobium* strain L-07 by addition of tritium-labelled retinal and subsequently reduced with cyanoborohydride. Lysis of the labelled vesicles in water and dissolution of the cell membranes with 4% SDS allowed the purification of the retinyl protein (RP) by a 3-step procedure. Gel filtration on AcA-44 ultrogel was followed by chromatography on hydroxylapatite and preparative SDS-polyacrylamide gel electrophoresis. This procedure yielded material which migrated as a single band of an apparent mol. wt. of 25 000 on analytical SDS-polyacrylamide gels. The purification was ~400-fold with an overall yield of ~15%. Not only the mol. wts. but also the amino acid compositions of the RPs from bacteriorhodopsin (BR) and HR are very similar. Polyclonal antibodies against BR and HR did not, however, crossreact. When the two RPs were partially digested with staphylococcal V8 protease the proteolytic pattern of the retinyl peptides was similar, but not identical: two extra peptides are present in BR. The same kind of differences were found in the h.p.l.c. elution profiles of retinyl peptides produced by subtilisin digestion. Therefore, the two proteins must be different gene products and not modification products of one and the same protein.

Key words: halorhodopsin/retinyl protein/light-driven ion pump/halobacteria/archaebacteria

Introduction

Halobacteria have the unique capacity of *de novo* synthesis of retinal. It is incorporated as a prosthetic group into bacterio-opsin, which is the dominant retinal-binding protein in some halobacterial strains and acts as a light-driven proton pump (Oesterhelt and Stoeckenius, 1973). Bacteriorhodopsin (BR) molecules are assembled in the cell membrane of the bacteria as a specialized area called the purple membrane. More recently, additional retinal-binding proteins involved in the photobioenergetics and phototaxis of halobacteria have been found, which compared to BR occur at much smaller concentrations in the cell membrane. One of these pigments originally described as a BR different from that of the purple membrane (Matsuno-Yagi and Mukohata, 1977) was functionally characterized as a light-driven sodium pump (Lindley and McDonald, 1979) and designated as halorhodopsin (HR) (Mukohata *et al.*, 1980). The evidence of its function was based mainly on the demonstration of light-induced, uncoupler-insensitive, sodium extrusion and enhanced passive proton uptake of cell envelope vesicles prepared from wild-type cells (Greene and Lanyi, 1979; MacDonald *et al.*, 1979; Matsuno-Yagi and Mukohata, 1980). Recently, additional evidence led Schobert and Lanyi (1982) to suggest that HR is an inward directed chloride pump rather than an outward directed sodium pump. Isolation of carotenoid-deficient mutants unable to synthesize BR considerably facilitated the spectrophotometric identification of HR (Lanyi and Weber, 1980) and description of its photochemical behaviour (Bogomolni *et al.*, 1981) thereby clearly demonstrating that its properties were distinct from those of BR. However, molecular properties could not be determined from these photophysical and photochemical studies and it is still not clear whether functional HR is a monomeric protein like BR or a multi-polypeptide complex. When strains became available which are retinal-negative but contain enhanced levels of the apoprotein of HR (Wagner *et al.*, 1981), the retinal-binding component of HR could be identified. Reconstitution with tritium-labelled retinal, reduction with cyanoborohydride, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single retinyl protein (RP) of apparent mol. wt. ~25 000 (Lanyi and Oesterhelt, 1982). The isolation of HR is a prerequisite for its functional reconstitution and detailed study of its mechanism as an ion pump. In this paper we report on the isolation of the retinal-binding component of HR in its reduced form and the comparison with the corresponding RP from BR.

Results

Preparation of radioactive-labelled HR, reduction of its chromophore, and preparation of cell membrane fractions

Limited aeration of *Halobacterium halobium* cultures induces BR synthesis. Maximal BR content in R_1M_1 cells is found after 50 – 70 h of growth (Oesterhelt and Stoeckenius, 1974). Table I shows that this is also true for the synthesis of HR in L-33. Here HR transport activity of cells of the L-33 culture rather than HR concentrations are given because the HR content is too low for direct spectrophotometric determination. Halo-opsin synthesis in L-07 cells is assumed to follow the same time course as found for the two other strains and cells therefore were harvested after 70 h of growth.

The most convenient way to prepare HR is reconstitution of the pigment with radioactive retinal in cell envelope vesicles of strain L-07. The vesicles are prepared by sonication of the cells and isolated by centrifugation. Typical yields are ~2 g of vesicle protein from a 15 l culture. [^{3}H]Retinal of high specific radioactivity was obtained by the simple reduction and reoxidation procedure described by Lanyi and Oesterhelt (1982). Reconstitution may be followed by difference spectroscopy (Lanyi and Weber, 1980; Lanyi and Oesterhelt, 1982) and by measurement of HR activity. The assay is uncoupler enhanced passive proton inflow upon light activation of HR as shown in Figure 1 (for a detailed description see Oesterhelt, 1982). The initial velocity of the pH change is proportional to the amount of HR reconstituted although the specific transport activity is <10% that of native HR. The vesicles typically contain ~1 nmol halo-opsin per 10 mg of protein.

Reduction of the reconstituted active HR is necessary for stabilization of the presumed Schiff's base linkage between retinal and the protein. As shown earlier (Lanyi and Oester-

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Table I. Induction of HR synthesis by limited aeration during growth.

Growth time (h)	16	22	38	43	48	62	67	71	86	91
Cell density (turbidity at 578 nm Eppendorf)	0.39	0.48	1.10	1.21	1.32	1.55	1.56	1.54	1.50	1.40
Vol. activity (ng H^+/min/ml cell-culture)	Not measurable	1.1	3.9	2.8	4.1	6.7	6.1	6.3	5.0	6.2
Rel. HR content (%)	–	16	58	42	61	100	91	94	75	93

Cells were grown under standard conditions (120 l air/h, 300 r.p.m. and 40°C) and 8 ml aliquots withdrawn at various times. The assay of HR was carried out as described (Oesterhelt, 1982) and calculated as volume activity. The relative HR content of the cells is given as percent of maximum.

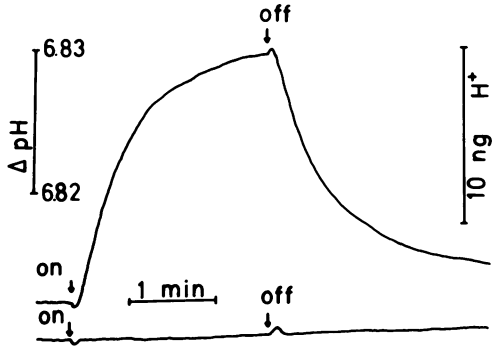

Fig. 1. Reconstitution of HR activity in vesicles of L-07 cells. Vesicles (2 mg) were incubated with (**B**) or without (**A**) 2 nmol all-*trans* retinal for 48 h and light-induced pH changes measured in the presence of 50 μM carboxylcyanide m-chloro-phenylhydrazone. Only the sample with retinal shows light-induced alkalinization.

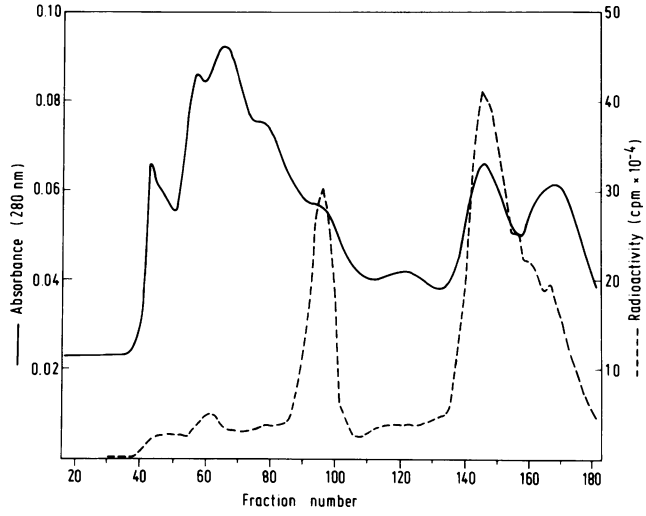

Fig. 2. Gel filtration of [^{3}H]retinal-labelled and reduced L-07 membrane fraction solubilized in 4% SDS on ultrogel AcA 44. Elution profile of 280 nm absorbing material (———) and radioactivity (– – – –). [^{3}H]RP elutes in fractions 90–100 and low mol. wt. compounds (mainly [^{3}H]retinol) after fraction 135.

helt, 1982), reduction of the pigment with cyanoborohydride in the presence of diethylether produces a high proportion of RP which can be used as a radioactive marker during purification. For preparative purposes reconstitution of HR with sub-stoichiometric amounts of retinal has the advantage of avoiding excess radioactivity which is not protein-bound. Apo-protein and RP, on the other hand, do not separate during purification. This will allow in the future the preparation of denatured retinal-free apo-protein, which in principle can be reconstituted, together with retinal, in liposomal systems as described for BR by Huang *et al.* (1981).

Isolation of the RP

After reconstitution and reduction the vesicles are lysed with water. The cell membrane fragments are sedimented by centrifugation and the pellet is dissolved in 8 M urea, 10% SDS, and 1% mercaptoethanol at pH 7. A high concentration of SDS is necessary for a quantitative solubilization of the cell membrane proteins and is complete only after stirring overnight or thorough homogenization in a Potter Elvejhem homogenizer.

The first step of purification is gel filtration on ultrogel AcA 44. The elution profile of protein is shown in Figure 2. The RP elutes as a narrow radioactive peak with an approximate K_d value of 0.5. It is separated from the bulk proteins which are generally larger and from low mol. wt. material which contains radioactive retinol and its degradation products. Radioactivity eluted at lower mol. wts. is due not only to free retinol arising from incomplete reconstitution and reduction of HR but also from slow degradation of the retinyl label attached to the protein. The extreme tendency of the RP to aggregate into oligomeric or polymeric forms (see also Figure 7, trace c) requires a high concentration of SDS (4%) and the omission of any buffer. In the presence of a buffer most of RP elutes with the exclusion volume. This aggregation can be explained by the influence of salt on the critical micellar concentration of SDS and on hydrophobic interactions in general (Helenius *et al.*, 1979). The combined RP-containing fractions from gel filtration are applied to a hydroxylapatite column and eluted under conditions described in Figure 3. Radioactive RP elutes between 300 and 500 mM sodium phosphate as a very broad zone from fractions 35–50. Both peaks in this zone contain RP, as shown by analytical SDS-PAGE, but elute separately on hydroxylapatite for unknown reasons. The main advantage of this step is the removal of proteins with mol. wts. very similar to RP which otherwise could not be removed. Stepwise elution which also can be used in hydroxylapatite chromatography does not yield reproducible results as obtained with gradient elution.

The last step in the purification is preparative SDS-PAGE. Electrophoresis is carried out essentially as described by

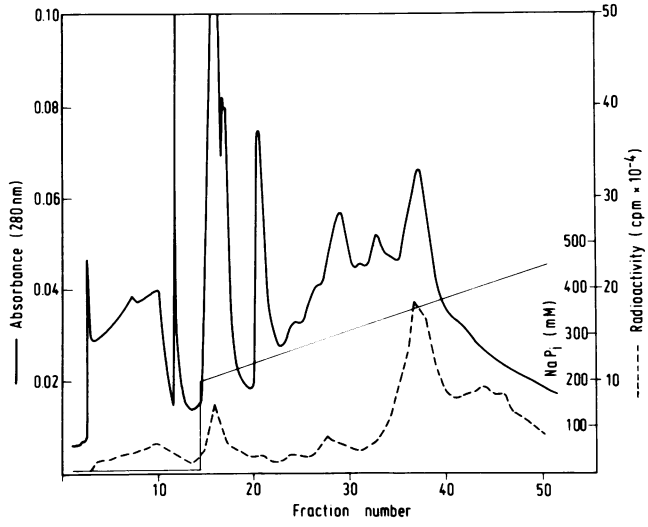

Fig. 3. SDS/hydroxylapatite chromatography (Biogel HT) of the pooled fractions 90 – 100 from the gel column (Figure 2). Elution profile of 280 nm absorbing material (———), radioactivity (– – – –) and the phosphate gradient are shown. The small radioactive peak at the start of the gradient is due to degradation products of [^{3}H]RP which is eluted in two broad radioactive peaks (frctions 35 – 50).

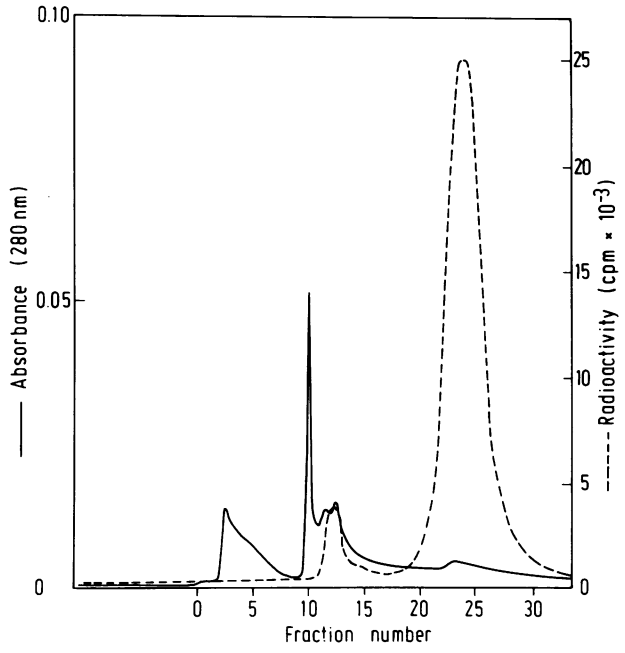

Fig. 4. Preparative SDS-PAGE of the pooled fractions 35 – 50 from the hydroxylapatite column on a LKB Uniphor-7900 apparatus. 280 nm absorbing material (———) in fractions 2 – 11 are contaminating substances from the gel (Righetti and Secchi, 1972). The first small radioactive (– – – –) peak is low mol. wt. radioactivity due to degradation of the [^{3}H]RP eluting in the main radioactive peak. Electrophoresis was stopped after collection of fraction 35.

Waehneldt (1971) but with 3 M urea included in the gel in order to avoid aggregation of the RP during electrophoresis. Figure 4 shows the elution pattern of radioactivity. The main peak is RP which follows a low mol. wt. radioactive peak resulting from decomposition of some retinyl label during electrophoresis. The trace of λ 280 nm absorption reveals only a small protein peak of RP and two non-protein peaks which are due to the indicator dye and some contaminating substances eluted from the gel. The rest of the proteins elute after fraction 30 in increasingly broader peaks and are not shown in Figure 4. The purified RP migrates as a single band positioned just below BR on SDS-PAGE (Figure 5, lane E and F). The protein mixture after hydroxylapatite chromatography (Figure 5, lane D) seems to be not very different from that after gel filtration (lane C) but without the hydroxylapatite step pure RP cannot be obtained. The crude membrane protein mixture is shown in lane B. It was overloaded with protein for visualization of RP in the mixture. Fluorography of the gel of Figure 5 shows that RP is the only radioactive protein band (see also Lanyi and Oesterhelt, 1982).

The apparent mol. wt. difference between reduced BR and reduced HR is ~1200 daltons. The apparent mol. wt. of BR on SDS-gel is 21 500 and is somewhat lower than its true mol. wt. (25 700 daltons). A reasonable assumption is that the hydrophobic character of BR is responsible for this anomalous behaviour and seems also appropriate for HR. We therefore assume that the mol. wt. of the RP from HR is close to 25 000 daltons. The purification factor of the procedure described is 400 with respect to cell envelope vesicles and >3000 with respect to total cellular protein. The yield is ~15%: the losses being mainly due to aggregation of RP.

Characterizaton of the RP from HR and comparison with the corresponding RP from BR

The similarity in mol. wt. suggests that one should check the possibility that the retinal-binding component in HR may be derived from BR by chemical modification or proteolytic processing during maturation. Thus, we first performed an amino acid analysis. For this purpose RPs from HR and BR were eluted from SDS-gels in order to remove residual impurities. As a result, the amino acid compositions were not significantly different from each other except for leucine which occurs at a lower concentration in the RP from HR than from BR. No N-terminal amino acid could be found, as is true for BR in which the N-terminus is blocked by pyroglutamic acid. This blocking residue can be removed by treatment of native purple membrane with pyroglutamate-aminopeptidase. However, it was not possible to unblock BR after SDS-PAGE. This is very likely due to traces of SDS which cannot be removed from proteins and might inhibit the pyroglutamate-aminopeptidase. We therefore can only conclude that RP from HR is N-terminally blocked as is BR. At this time we do not know whether this is due to a pyroglutamic acid residue as in BR or to another chemical modification.

The degree of purification attained made it possible to prepare specific antibodies against the RPs of both BR and HR. This was done with a standard program of immunization and injection of the pure proteins into rabbits. The titer of antibodies attained permitted a serum dilution of 1:25 to 1:50, when used in antibody stain experiments. In these tests, radioactive samples of the RPs from BR and HR were run as four separate sets on SDS-PAGE and then transferred to nitrocellulose by the electrophoretic blotting procedure of Towbin *et al.* (1979). The first set was stained on the gel with Coomassie blue R 250, the second and third set on the nitrocellulose exposed to antibodies against RPs from BR or HR and stained afterwards with fluoresceine-conjugated goat antibodies against rabbit immunoglobulins. The fourth set was used to expose an X-ray film to check the efficiency of protein-transfer to the nitrocellulose. As can be seen from Figure 6, antibodies against BR did not react with HR. Conversely, no reaction of antibodies against HR could be

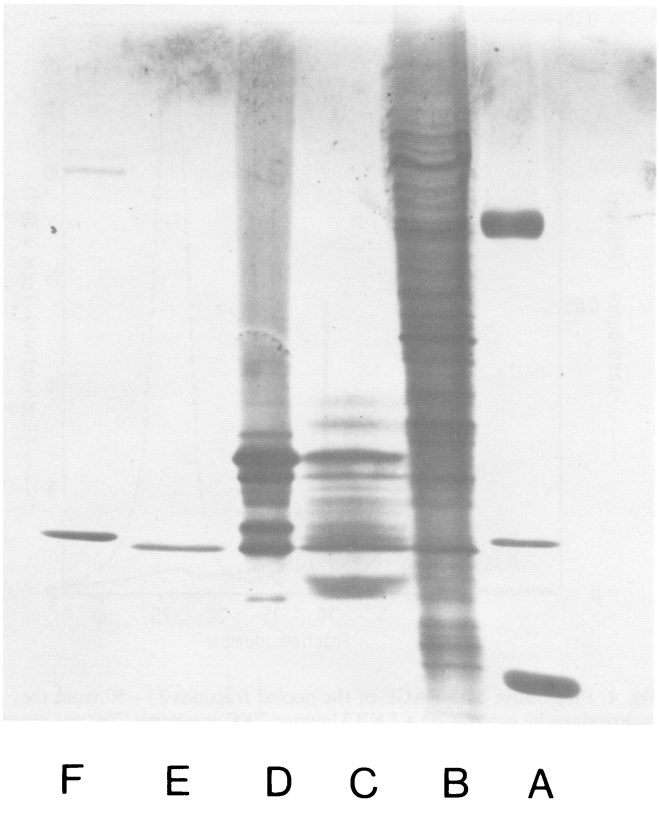

Fig. 5. Analytical SDS-PAGE in 3 M urea of the RP-containing fractions from the various stages of purification. The gel was stained with Coomassie blue R250. **Lane A**: marker proteins (bovine serum albumin 68 000 daltons, BR 25 700, cytochrome *c* 12 500); **lane B**: lysate of labelled cell vesicle after centrifugation (cell membrane fraction); **lane C**: pooled fractions 90 – 100 after gel filtration; **lane D**: pooled fractions 35 – 50 after hydroxylapatite chromatography; **lane E**: RP after preparative SDS-PAGE; **lane F**: BR.

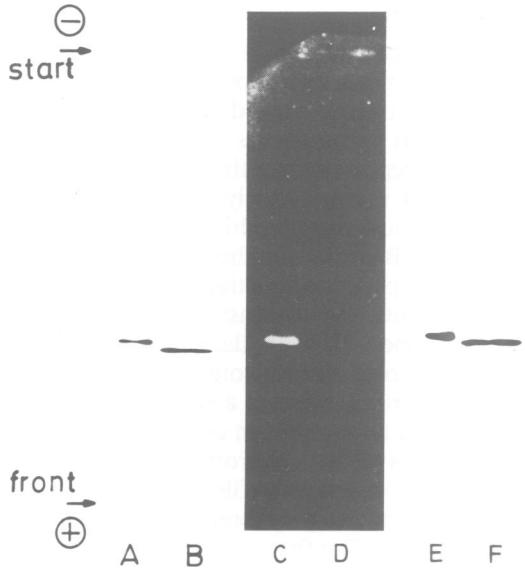

Fig. 6. Immunological differentiation between the RPs from BR and HR. **Lane A** and **B**: Coomassie stain of BR (**A**) and HR (**B**); **lane C** and **D**: fluorescence pattern on nitrocellulose after transfer from the gel and incubation with BR-antiserum of rabbit and fluoresceine-conjugated anti rabbit IgG from goat. Note that **lane D** has no stain; **lane E** and **F**: fluorographs of the [^{3}H]RP after transfer to nitrocellulose.

observed with BR (not shown). Therefore, different antigenic determinants must be present in BR and HR although this does not exclude the presence of common sequences in the primary structure of the two proteins.

A structural comparison of HR and BR is further possible by comparing limited proteolysis patterns of the two proteins. Staphylococcal V8 protease in ammonium carbonate buffer cleaves polypeptide chains only at the carboxyl end of glutamic acid residues except Glu-Asp and at Asp-Gly bonds (Hoummard and Drapeau, 1972). The enzyme is also active in the presence of SDS (Cleveland *et al.*, 1977). When used under identical conditions of incubation this enzyme allows a comparison of the retinyl peptide patterns obtained from BR and HR as shown in Figure 7. Four fragments of different size are produced from BR with approximate apparent mol. wts. of 22 500, 17 800, 14 600, and 10 000. This can be interpreted as a limited proteolysis at the residues Glu-9, Glu-74, Asp-Gly-115 (and possibly Asp-Gly-243), Glu-161, and Glu-232. When proteolysis is carried out to completion only fragments around mol. wt. 7000 are found. Proteolysis of HR produces two fragments which have mol. wts. similar to the largest and the smallest piece from BR. This result suggests similarities as well as differences in the primary structure of both proteins. The similarity is that from the two polypeptide RP chains, which have different mol. wts., two fragments of similar size are cut out. On the other hand, the two fragments absent in the pattern of RP from HR imply that either differences in the primary structure do not allow a cut by the protease around the positions Glu-74 and Asp-115 or that in RP from HR no such residues occur around these positions. To demonstrate further the differences between the two RPs, we compared the retinyl peptides using h.p.l.c. Both RPs were subjected to subtilisin digestion after addition of carrier RP from BR (Lemke and Oesterhelt, 1981). The chromatogram in Figure 8 clearly shows differences between the elution profiles of the radioactive retinyl peptides from BR and HR.

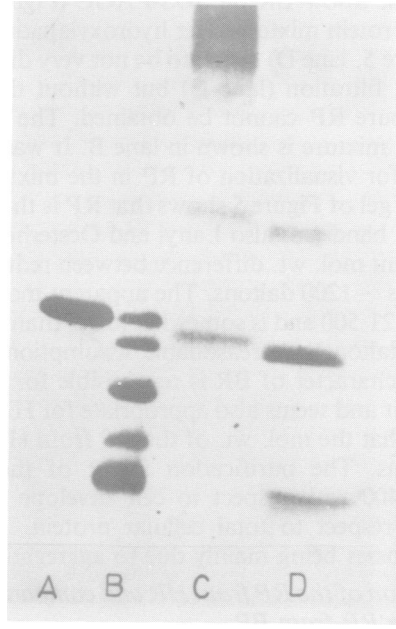

Fig. 7. Fluorography of the [^{3}H]RP from BR and HR after limited proteolysis with staphylococcal V8 protease and SDS-PAGE. **Lane A** and **B**: [^{3}H]retinyl-opsin before (**A**) and after (**B**) protease treatment; **lane C** and **D**: [^{3}H]RP from HR before (**C**) and after (**D**) the same treatment.

The main peptide from BR elutes at 6 min whereas the main peptide from HR elutes at 9 min. In addition to the radioactive elution pattern the elution profile of material absorbing at 328 nm is shown. This absorbance is characteristic of retinyl residues of the carrier RP from BR, and should correlate with the radioactive pattern of the retinyl peptide from BR. The imperfect correlation seen in Figure 8 might be because peptides rich in tryptophan show residual absorption at 328 nm and thereby interfere with the retinyl peptide pattern.

Discussion

BR can easily be isolated as a native pigment because it aggregates into the crystalline arrays of purple membranes, which may cover >50% of the total cell membrane area. Lysis of cells and membrane fractionation by sucrose density centrifugation yields large amounts of pure material. In contrast, HR occurs at ~100-fold lower concentrations in the cell membrane and does not enrich into a specific membrane fraction of the wild-type cells. The search for mutants unable to produce BR but synthesizing enhanced levels of HR resulted in the selection of the stable mutant strain L-33. However, even after optimization of growth conditions for HR synthesis in these cells, which proved to be the same as for maximum production of BR in the wild-type cells, the HR was not associated specifically with a distinct membrane fraction. Furthermore, and unlike BR, the chromophore of HR becomes easily decoloured upon solubilization of the membranes with detergents and attempts to purify HR in the native state have failed so far. Therefore, in retinal-deficient L-07 cells we labelled the protein with radioactive retinal and stabilized the linkage between retinal and protein by reduction. Although reconstitution of HR and reduction are not quantitative under our conditions, the label is specific for HR and stable enough to allow complete purification. The apoprotein co-purifies with the RPs and this will allow its isolation also from L-33 cells, which produce larger amounts of HR than L-07 cells. The isolated apoprotein can then be used for reconstitution experiments of HR in liposomal systems. In principle, it will be possible not only to reconstitute the chromoprotein but also to check on its bioenergetic function.

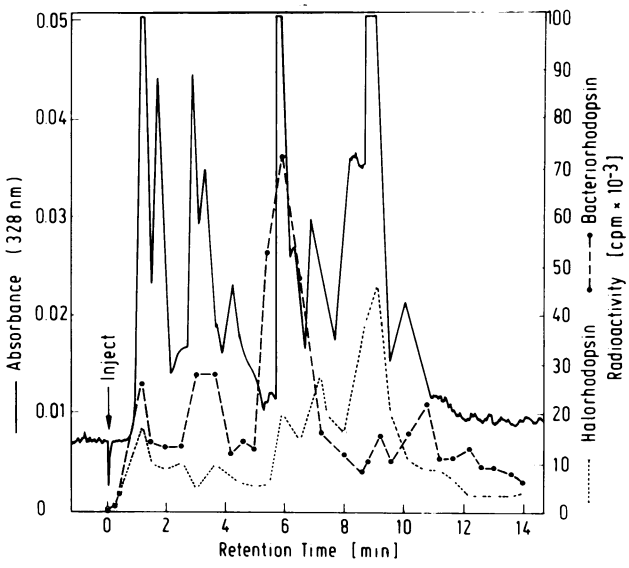

Fig. 8. H.p.l.c. of ethanol-soluble retinyl peptides after subtilisin cleavage of RPs: ———, carrier retinyl peptides from BR detected by absorption at 328 nm; – – – –, [^{3}H]retinyl peptides from BR; • • • •, [^{3}H]retinyl peptides from HR.

The comparison of the RPs from BR and HR revealed surprising similarities. The RPs cannot be separated during the purification procedure and this makes the use of a strictly BR-negative mutant necessary. While co-migration of the RPs is expected for the separation steps based on mol. wt., co-elution from hydroxylapatite indicates similar adsorption properties. Further similarities are the amino acid composition, the blocked N-termini, and the limited proteolysis pattern. This, on the other hand, also demonstrates that at specific places in the polypeptide chain amino acid exchange must have taken place. Given these similarities it seems surprising that antibodies against HR and BR did not cross-react. Immunization of rabbits or mice with the intact polypeptide chain of BR for production of polyclonal or monoclonal antibodies revealed that only a few antigenic determinants are active (Kimura *et al.*, 1982). Assuming that the same properties are shown by HR this might explain why our polyclonal antibodies against both proteins do not cross-react although their primary sequences might be very similar. When the polypeptide chain of BR is cleaved with cyanogen bromide and the separated fragments are coupled to a carrier protein and used for immunization, antibodies are produced which react with both BR and HR, thereby confirming that both proteins share common sequences (Lemke and Oesterhelt, unpublished results). The antibodies against HR will be very useful for affinity chromatography of the native pigment and also for isolation of HR-specific peptide sequences. The elucidation of such sequences will be a prerequisite for gene isolation.

The isolation of the polypeptide chain of the retinal-binding component of HR will also help us tackle the fascinating problem of how two retinal-binding proteins of great similarity can catalyse the light-induced translocation of different ions, such as protons and sodium ions. Three possibilities exist for the action of HR as an ion pump. (1) The functional retinal protein consists of one polypeptide chain and the differences in primary sequence between HR and BR can account for a change in ion specificity. (2) The retinal protein of HR is associated with another polypeptide chain or chains and drives an internal cycle of protons within such a complex. The catalytic activity of the other components of the complex would then be a conversion of the electrochemical energy of the protons into that of other ions. As an example, a light-driven sodium pump would arise from a molecular association and energetic coupling of a retinal-binding protein with an H^+/Na^+ antiporter. If HR functions as a chloride pump as suggested recently by Schobert and Lanyi (1982) it could consist of the retinal-binding protein associated with a Cl^-/OH^- antiporter. However, before these problems can be approached experimentally the primary sequence of the retinal protein from HR will have to be elucidated and reconstitution of the chromoprotein must be possible.

Materials and methods

Growth of bacteria and preparation of cell vesicles

Growth of *H. halobium* L-07 and L-33 cells was carried out under limited aeration as described previously (Wagner *et al.*, 1982; Oesterhelt and Stoeckenius, 1974; Oesterhelt, 1982). Since halo-opsin synthesis cannot be monitored directly in L-07 cells, conditions for maximal production were approximated based on those for HR synthesis in L-33 cells grown under identical conditions. Aliquots of 8 ml were withdrawn from the fermenter vessel

and spun down at 10 000 *g* for 10 min. After resuspension in 8 ml basal salt (medium without peptone and citrate) the HR activity was determined as described (Oesterhelt, 1982). Maximal activity was found after 70 h growth. Therefore, L-07 cells were harvested after this time by centrifugation for 90 min at 3000 *g* (Stock centrifuge, Marburg). Cells from 15 l culture medium were resuspended in 300 ml of basal salt, washed with 4 M NaCl, and centrifuged at 14 000 *g* for 15 min. After resuspension in 300 ml 4 M NaCl 150 ml aliquots of the suspension were sonicated in an ice-bath using a sonication horn and the macrotip of a Branson sonifier B15. The 50% pulse mode at level 9 for 6 min was used. Cell disintegration was checked by microscopic inspection. After centrifugation at 14 000 *g* for 15 min, the supernatant was centrifuged at 200 000 *g* for 1 h. The pellet was washed once with 300 ml 4 M NaCl and then resuspended in 20 ml 4 M NaCl. The protein concentration was determined (Warburg and Christian, 1942) and adjusted to 50 mg/ml. The protein yield at this step was ~2 g, the approximate halo-opsin content was ~1.0 nmol/10 mg of protein. This value can be determined by spectroscopic titration of an aliquot with retinal (Lanyi and Oesterhelt, 1982).

Reconstitution of HR and lysis of cell vesicles

To the vesicle suspension, 0.5 nmol [^{3}H]C_{15}-retinal (5 Ci/mmol) per 10 mg of protein were added in methanol solution (~1 mM) at room temperature and the suspension stirred in the dark overnight. Then reduction of the reconstituted HR was carried out as described previously (Lanyi and Oesterhelt, 1982) at a cyanoborohydride concentration of 2%. The vesicles were then washed twice with 4 M NaCl (200 000 *g*, 1 h), resuspended in 40 ml 4 M NaCl containing 5 mg DNase I (bovine pancreas grade II, Boehringer) and dialysed against 10 l of water overnight. After dialysis, an additional 5 mg of DNase were added and the suspension stirred for 1 h at room temperature. After centrifugation (200 000 *g*, 1 h) the pellet containing the total membrane fraction was washed twice with water and the protein content determined before the second centrifugation. Usually 1 – 1.5 g of total protein are obtained. The membrane pellet was resuspended in a buffer containing 8 M urea, 10% SDS, 1% mercaptoethanol, 5 mM EDTA, 10 mM sodium phosphate pH 7, which was prepared and stored at room temperature. Resuspension was achieved by stirring overnight or by homogenization in a Potter Elvejhem homogenizer and subsequent stirring for 3 h. As the suspension progressed it became clear and was centrifuged at 200 000 *g* for 1 h. This allowed removal of insoluble debris present in the preparation.

Gel filtration

The supernatant (15 ml) of the last centrifugation step was applied to the top of a 5 x 90 cm column filled with AcA 44 ultrogel (LKB) equilibrated with 4% SDS in 1 mM EDTA filtered through a 0.25 μm Millipore membrane (see also Steffens and Buse, 1976). The material was eluted at a hydrostatic pressure of 90 cm which gave a rate of elution of 1.6 ml/min. Fractions of 12 ml were collected and radioactivity measured. The RP eluted before low mol. wt. radioactive compounds which were present as a result of incomplete reconstitution.

Hydroxylapatite chromatography

SDS-hydroxylapatite chromatography was carried out at constant temperature (22°C) essentially as described (Moss and Rosenblum, 1972). The RP-containing fractions from the gel column were combined and applied to a hydroxylapatite column (1.6 x 20 cm) filled with biogel HT (Bio-Rad) equilibrated with 10 mM sodium phosphate pH 6.4, 0.1% SDS, 0.01% sodium azide, and 1 mM EDTA. Equilibration was with three times the column volume. After application of the sample, the column was first washed with 30 ml of the equlibration buffer and then the protein was eluted with a gradient from 200 to 500 mM sodium phosphate at a rate of 0.5 ml/min and 10 ml fractions were collected. RP-containing fractions were combined, dialysed against 1 mM EDTA at 4°C and then concentrated in an Amicon cell (YM-10 filter) to a final volume of 500 μl.

Preparative SDS-PAGE

Preparative SDS-PAGE was carried out according to Waehneldt (1971) with an LKB Uniphor 7900. The concentrated sample (10 mg protein, 500 μl total volume) was applied in buffer, which contained 4% SDS, 8 M urea, 15 mM Tris-HCl pH 7.8, 2% mercaptoethanol, 10% sucrose, and 0.01% bromophenol blue. The separating gel contained 12% acrylamide and 0.32% bisacrylamide. The gel was 10 cm in length and the inner diameter of the column was 2.2 cm. The stacking gel consisted of 2.5% acrylamide and 0.62% bisacrylamide and had a height of 1 cm. The preparation of the gels were exactly as described by Waehneldt but included addition of urea to a final concentration of 3 M in both gels. The cathode buffer consisted of 50 mM Tris/glycine pH 8.9 in 0.1% SDS, the anode buffer and the eluting buffer contained 100 mM Tris/HCl pH 8.1 in 0.1% SDS. Electrophoresis was carried out for 3 h at 6 mA (200 V) followed by a period at 20 h with a constant current at 10 mA (the voltage increased up to 600 V). The speed of elution was 0.2 ml/min and fractions of 5 ml were collected. Radioactive fractions were combined, dialysed and concentrated as described for the hydroxylapatite column eluate.

Preparation of antibodies

BR was isolated as purple membranes from strain R_1M_1 or S9 as described (Oesterhelt and Stoeckenius, 1974) and subjected to hydroxylapatite chromatography as described for HR. Antibodies against BR were raised in rabbits by injection of 550 μg of the hydroxylapatite purified protein emulsified with complete Freund's adjuvant s.c. at three sites in each animal. Purified HR (400 μg) was injected by the same method and booster injections with incomplete Freund's adjuvant and 200 μg of protein were given 4 weeks after the first injection. This was repeated weekly for four additional weeks. The serums obtained could be used at a dilution of 1:25 – 1:50.

Analytical procedures

Protein fractions containing the RP or the pure proteins were analyzed on gradient (9–24%) SDS-PAGE according to Laemmli (1970). The gel contained 3 M urea. The proteins were transferred to nitrocellulose by the procedure described by Towbin *et al.* (1979), at a voltage of 30 V and a current of 200 – 300 mA for 4 h. As indicator antibodies we used fluorescamine-conjugated goat anti-rabbit IgG (Bio Merieux) at a 1:100 dilution. Residual protein on the gels was stained with Coomassie blue R 250. Protein on nitrocellulose was either stained with amido black or immunologically as described above (Towbin *et al.*, 1979).

Limited proteolysis

20 μg of electrophoretically pure RP from BR or HR were dissolved in 10 μl of enzyme buffer (0.125 M Tris/HCl pH 6.8, 0.5% SDS, 10% glycerol, 0.0001% bromophenol blue) and 30 μl of a *Staphylococcus aureus* V8 protease solution (0.5 mg/ml) added. After incubation at 30°C for 1 h, 10 μl of a stop solution (10% mercaptoethanol, 20% SDS) were added and the mixture heated for 5 min to 95°C. The samples were then applied directly to analytical SDS-PAGE.

Fluorography

After electrophoresis the gels were impregnated with a liquid scintillant (En^{3}Hance, New England Nuclear) dried on a filter paper sheet (Whatman 3MM) and used to expose (1 week for 200 000 c.p.m.) at –70°C a Kodak X-ray 90 film. Nitrocellulose sheets were impregnated for 30 min with 1 M salicylate before exposure of an X-ray film.

Chromatography of retinyl peptides

BR was reconstituted with retinal and the RP prepared as described (Oesterhelt and Schuhmann, 1974). 200 nmol of RP from BR were disoslved in 2 ml of 10% SDS, 20 nmol of labelled RP from BR or HR added and the samples dialyzed overnight against 10 l of 1 mM EDTA pH 7. After concentration of the dialysate to 2 ml in an Amicon cell (YM10-filter) the sample was lyophilized and the RP extracted from SDS by the ionpair method A of Henderson *et al.* (1979). They were then suspended in 1.5 ml of a 0.1 M n-methylmorpholine acetate buffer pH 7.5 by sonication (microtip of a Branson sonifier with a pulse mode of 50/1 min at level 7), subtilisin (5% w:w) was added and the sample digested at 37°C for 4 h. After lyophilisation the retinyl peptides were extracted with ethanol and separated on a h.p.l.c. column packed with Merck Si-60 (7 μm, column size 4.6 x 250 mm, Gynkothek pump, 600/200, Kontron Uvicord 725 flow photometer) using chloroform/methanol/ammonia (70:27:3) as solvent. The elution rate was 2 ml/min. The carrier peptides were detected by the retinyl absorption at 328 nm. Fractions of 1 ml were collected and monitored for radioactivity after drying *in vacuo* and solubilization of the residue in Unisolve (Zinsser, Frankfurt, FRG).

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