Bacteriorhodopsin Precursor
CHARACTERIZATION AND ITS INTEGRATION INTO THE PURPLE MEMBRANE*

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Halobacterium halobium spheroplasts synthesize and accumulate a bacteriorhodopsin precursor. By labeling of the precursor with [35S]Met and [3H]Leu followed by Edman degradation, we have confirmed the previous conclusion from the DNA sequencing that the precursor contains 13 additional amino acids at the NH₂ terminus of bacteriorhodopsin. Although not processed in the spheroplasts, it integrates into the purple membrane in the correctly folded conformation. This was shown by the mode of cleavage by a number of proteolytic enzymes, the site of attachment of retinal, and the formation of oligomers on reaction with bifunctional cross-linking reagents. In all these respects, the behavior of the precursor was identical with that of native mature bacteriorhodopsin in the purple membrane. Finally, the precursor was not processed to bacteriorhodopsin even when the spheroplasts were subsequently allowed to revert to rod-shaped cells. This suggests that either the processing of the precursor is cotranslational or that the NH₂ terminus of the precursor becomes inaccessible to the processing enzyme in the spheroplasts following integration into the membrane.

The purple membrane of the extremely halophilic bacterium Halobacterium halobium is a specialized membrane that carries out light-dependent proton translocation (1, 2). BR, the only protein present in this membrane, consists of a single polypeptide chain of 248 amino acids. Its amino acid sequence has been determined both by protein sequencing (3, 4) and by gene sequencing (5). One molecule of retinal bound to lysine 216 as a Schiff's base serves as the chromophore (6-8). A secondary structure model (Fig. 1A) has been proposed for BR that attempts to accommodate the electron and neutron diffraction data (9, 10), the amino acid sequence, and the cross-linking data (11).

Little is known about the synthesis and the mode of insertion and folding of BR into the cytoplasmic membrane of H. halobium. Dellweg and Sumper (12) detected a precursor, pre-BR, which was subsequently identified by Chang et al. (13) by sequencing a cDNA fragment prepared from BR mRNA. The precursor sequence (Fig. 1B) was very different from the usual NH₂-terminal signal sequences of the soluble secretory proteins (14-16). Thus, it contained only 13 amino acids, and among these, there were only six contiguous neutral or hydrophobic amino acids, and these were flanked by glutamic acid residues at both ends.

To obtain some insight into the role of pre-BR in the insertion and folding of BR within the H. halobium cytoplasmic membrane, we have now carried out a further study of its formation and properties. We used the Mg²⁺ starvation procedure (12), which converts the normal rod-shaped H. halobium cells to spheroplasts that lack the glycoprotein coat. Pulse labeling with [35S]Met produced 35S-labeled pre-BR. The precursor copurified with the PM, which normally contains the mature BR. Amino acid sequence analysis of the labeled protein accompanying the PM confirmed its identity as pre-BR, the amino acid terminal sequence being in complete agreement with that encoded in the BR mRNA. We showed next, by a number of criteria normally applied to native folded BR, that pre-BR, while not processed in the spheroplasts, integrates into PM and acquires the tertiary structure characteristic of BR. We further show that when spheroplasts containing pre-BR are allowed to revert to rod-shaped cells, synthesis and processing of BR again occur as in the normal cells but pre-BR made at the spheroplast stage remains unprocessed.

EXPERIMENTAL PROCEDURES

Materials

[35S]Methionine was purchased from Amer sham Corp. [3H]Leucine was purchased from New England Nuclear. Bacterial protease (Streptomyces griseus), type VI, α-chymotrypsin, L-alanine, and phenylmethylsulfonyl fluoride were supplied by Sigma. Staphylococcal aureus protease was from Miles Laboratories, and Pronase was from Boehringer Mannheim.

Methods

Growth of H. halobium (15)—H. halobium, strain S9, was grown in peptone medium at 37 °C (doubling time, 14–18 h), and the cells were harvested in the midlog phase (turbidity ~0.7–1.0 units at 578 nm) by centrifugation (7000 × g) at 30 °C. They were washed with the basal salt solution that contained, per liter, 250 g of NaCl, 20 g of MgSO₄·7H₂O, and 2 g of KCl. Preparation of Spheroplasts and Pulse Labeling of Pre-BR (16)—Washed cells from a 25-ml culture grown as described above were suspended in 1 ml of 4 M NaCl containing 25 mM KCl and 5 g/liter of L-alanine (pH 7.3) as the carbon source. EDTA (0.25 mM of 0.5 M, pH 7.5) was then added, and the suspension was incubated for 20 min at 37 °C. Examination by phase-contrast microscopy showed that the conversion of the rod-shaped cells to spheroplasts was complete at this time. A solution (1–5 ml) containing 4 M NaCl and 1 M MgCl₂ was added; the cells were pelleted by centrifugation (6000 × g), and the pellet was resuspended in the basal salt solution (1 ml) containing L-alanine. [35S]Methionine (250 μCi) was added, and the cells were incubated at 37 °C for 3 h to label the proteins. The cells were then...
A. Bacteriorhodopsin

**INSIDE**

![Inside structure diagram]

**OUTSIDE**

![Outside structure diagram]

**Fig. 1.** A model for the secondary structure of bacteriorhodopsin (A) and the amino acid sequence in the precursor region of BR as derived from the DNA sequence (B). The site of cleavage by the putative peptidase is shown. The NH$_2$-terminal pyroglutamyl residue in mature BR is thus generated.

B. Precursor Sequence

\[ \text{H}_2\text{N}-\text{Met Leu Glu Leu Leu Pro Thr Ala Val Glu Gly Val Ser Gin Ala Gin Ile Thr Gly Arg } \ldots (\text{Bacteriorhodopsin}) \]

Peptidase

**Fig. 1.** The site of cleavage by the putative peptidase is shown. The NH$_2$-terminal pyroglutamyl residue in mature BR is thus generated.

Centrifuged (20,000 × g for 30 min) and washed with 4 M NaCl. The pelleted cells were lysed by suspension in distilled water, and the membranous fraction was collected by centrifugation at 30,000 × g for 30 min and washed with distilled water (3 × 20 ml) (17). After the final wash, the pellet was suspended by sonication (bath sonicator) in 1 ml of 50 mM Tris-HCl (pH 7.5) and the suspension was layered onto a sucrose density gradient (15-65% (w/v), 25 ml) and centrifuged at 150,000 × g for 16 h. The band of PM was isolated and washed with distilled water (4 × 25 ml) to remove the sucrose.

**Preparation of Pre-BR for Sequence Analysis—**Lyophilized PM containing pre-BR labeled with [35S]methionine or [3H]leucine was dissolved in formic acid, and the suspension was diluted with ethanol to 30% formic acid (v/v). The solution was applied to a column (120 × 0.7 cm) of Sephadex LH-60 equilibrated with formic acid/ethanol (3:7, v/v). Elution was carried out using the same solvent, and fractions containing radioactivity were pooled and dried in vacuo (0.05 mm Hg).

**Sequence Analysis of Pre-BR—**Pre-BR labeled with [35S]methionine or [3H]leucine (as described above) was sequenced in a Beckman 890C Sequencer using a modified 0.2 mmol/liter Na$_2$琵酸 (pH 5.4), 1.0% acetonitrile, and 28% MeOH (17, 19). Phenylthiohydantoin amino acids were monitored by their absorbance at 269 nm using a Kratos/Quadrol program with Polybrene added to the spinning cup to prevent washout of the peptide (17).

**In Vivo Processing of Pre-BR—**Cells were harvested during the midlog phase, and they were converted to spheroplasts. The latter were labeled with [35S]methionine (250 μCi) for 3 h, and after two washes with basal salts containing 0.1 mM MgCl₂, the cells were suspended in the growth medium containing peptone (volume same as during the original growth). The cells were grown for 2 days and harvested, and the pellet was suspended in basal salt containing 0.1 mM MgCl₂ and 5 g/liter of L-alanine. [3H]Leucine (250 μCi) was added, and the cells were incubated at 37°C for 1 h. The PM from these double-labeled cells was isolated as described above and analyzed by SDS-PAGE (20).

**Treatment of Pre-BR with Chymotrypsin (18)—**The PM containing pre-BR labeled and washed with 4 M NaCl. The pelleted cells were lysed by suspension in distilled water, and the membranous fraction was collected by centrifugation at 30,000 × g for 30 min and washed with distilled water (3 × 20 ml). After the final wash, the pellet was suspended by sonication (bath sonicator) in 1 ml of 50 mM Tris-HCl (pH 7.5) and the suspension was layered onto a sucrose density gradient (15-65% (w/v), 25 ml) and centrifuged at 150,000 × g for 16 h. The band of PM was isolated and washed with distilled water (4 × 25 ml) to remove the sucrose.

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**In Vivo Processing of Pre-BR—**Cells were harvested during the midlog phase, and they were converted to spheroplasts. The latter were labeled with [35S]methionine (250 μCi) for 3 h, and after two washes with basal salts containing 0.1 mM MgCl₂, the cells were suspended in the growth medium containing peptone (volume same as during the original growth). The cells were grown for 2 days and harvested, and the pellet was suspended in basal salt containing 0.1 mM MgCl₂ and 5 g/liter of L-alanine. [3H]Leucine (250 μCi) was added, and the cells were incubated at 37°C for 1 h. The PM from these double-labeled cells was isolated as described above and analyzed by SDS-PAGE (20).

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at 115,000 \( \times \) g for 3 h. The pellet was resuspended by gentle homogenization in 25 mM Tris-HCl (pH 7.5) containing 10 mM CaCl₂.

The cell envelopes were subjected to proteolysis by chymotrypsin as described above. Additionally, the cell envelopes were bleached in the presence of 3 \( \times \) M hydroxylamine HCl (pH 7.5) at 4 °C, and after washing, the envelopes were treated with chymotrypsin as described above. After proteolysis, the membranes were washed three times with H₂O and analyzed by SDS-PAGE using 8 M urea (21).

**Proteolysis by Bacterial Protease (S. griseus)**—Cell envelopes (3 mg) were suspended in 1 ml of Tris-HCl (25 mM, pH 7.5) containing 25 mM MgCl₂, bacterial protease (S. griseus) was added (membrane/protease ratio, 25:1), and the mixture was incubated at room temperature for 2 h. Proteolysis was stopped by the addition of EDTA (pH 7.5) to a final concentration of 100 mM, and the membranes were collected by centrifugation (40,000 \( \times \) g) and, after being washed several times with water, were analyzed by SDS-PAGE.

In another experiment, the cell envelopes were subjected to proteolysis as described above except that the incubation mixture was sonicated after the addition of protease, thus exposing the inner surface of the cell envelopes to proteolysis.

**Binding of Retinal to Pre-BR**—PM containing \(^{35}\)S-labeled pre-BR was bleached in the presence of 3 \( \times \) M hydroxylamine HCl (pH 7.5) as described above and washed with bovine serum albumin (24) to remove residual oxime. The apomembrane was regenerated by the addition of [\(^{3}H\)retinal (specific activity 1.1 \( \times \) 10⁶ dpm/nmol) in a ratio of 2 mol of retinal:1 mol of bacterio-opsin (6, 24). The regenerated membrane was washed and collected by centrifugation, and after resuspension in Tris-HCl (25 mM, pH 7.5), it was treated with NaBH₄, either in the presence of light or in total darkness as previously described (6). After 6 h, excess NaBH₄ was destroyed by the addition of 1 M acetic acid and the membrane was collected by centrifugation. The washed membrane was subjected to preparative SDS-PAGE (20), and the \(^{35}\)S-methionine-labeled precursor was isolated by electrophoresis and analyzed again by analytical SDS-PAGE. The labeled pre-BR containing reduced Schiff’s base was further purified on a column of Sephadex LH-60 that was developed in formic acid/ethanol (3:7). The precursor (10⁶ dpm of \(^{35}\)S-methionine) was dissolved in 70% formic acid containing 1 mg of delipidated bacterio-opsin, CNBr (3 mg) was added, and the reaction mixture was allowed to stand in the dark for 24 h. At the end of the incubation, the sample was vacuum-dried and redissolved in formic acid (6.9 ml) containing SDS (10 mg), and the solution was vacuum-dried. The dried pellet was redissolved in 250 \( \mu \)l of H₂O, and the pH was adjusted to 6.8 by the addition of Na₃HPO₄ (0.1 M). The dissolved sample was made 4 M in urea and analyzed by SDS-PAGE.

Cross-linking with Dimethyl-3,3’-dithiobispimoniimide—Cross-linking was performed using the conditions described by Dellweg and Sumper (25). FM containing bacteriorhodopsin (26 mg) and pre-BR (5 \( \times \) 10⁶ dpm) was suspended in 100 \( \mu \)l of 0.2 M N-methylmorpholine acetate buffer, pH 8.0. Dimethyl-3,3’-dithiobispimoniimide, 2.5 mg in 100 \( \mu \)l of 0.2 M N-methylmorpholine acetate (pH 8.0), was added, and the reaction mixture was incubated at 37 °C for 3 h. The products were analyzed by SDS-PAGE.

**Proteolysis by S. aureus Protease**—PM (1 mg) containing pre-BR (\[^{35}\]S-Met to \[^{3}H\]Leu ratio 1:10) was incubated in the presence of S. aureus protease (25 µg) using 50 mM ammonium bicarbonate buffer (pH 7.8) (26) in a final volume of 1 ml. Incubation was carried out at 37 °C for 3 h, and the \(^{3}H/^{35}\)S ratio in pre-BR was monitored after pelleting the purple membrane by centrifugation. Purple membrane was delipidated by column chromatography (Sephadex LH-60 in formic acid/ethanol, 3:7), and the NH₂-terminal sequence was determined.

**RESULTS**

**H. halobium Spheroplasts Synthesize but Do Not Process pre-BR**

**Pre-BR**

**H. halobium** cells were grown and converted to spheroplasts by Mg²⁺ starvation, and the latter were pulse-labeled with \[^{35}\]S-Met (see “Methods”). The PM was isolated from the spheroplasts and examined by SDS-PAGE (Fig. 2). Pre-BR (lane 2) migrating slower than BR (lane 3) was the only species labeled. The result also showed that the processing of pre-BR to the mature BR was totally inhibited in the spheroplasts.

**NH₂-terminal Amino Acid Sequence of the Pre-BR**

For sequencing, radioactively labeled preparations of pre-BR were made as follows. Spheroplasts were incubated (see “Methods”) in the presence of \[^{35}\]S-methionine or \[^{3}H\]leucine, and the PM was isolated. After delipidation of the PM, the protein was subjected to automated Edman degradation. The presence of the large amount of mature BR in PM did not interfere because the NH₂ terminus in BR is blocked (pyroglutamic acid) (18). The results (Fig. 3) show that the radio-
activity in [35S]Met appeared in cycle 1, while 3H present in Leu appeared in cycles 2, 4, and 5 and also in cycles 24 and 26. The results are in agreement with the precursor sequence derived from the nucleotide sequence of the BR gene. They also confirm that the precursor sequence at the NH2 terminus is 13 amino acids long since [3H]Leu was observed in cycles 24 and 26.

Pre-BR Remains Unprocessed When Spheroplasts Revert to Cells and Synthesize New Mature BR

The inability of the *H. halobium* spheroplasts to process pre-BR to the mature BR may be due to the loss of an appropriate peptidase or some component. Reconversion of the spheroplasts to rod-shaped cells might reverse this loss and result in processing of pre-BR to the mature BR.

*H. halobium* spheroplasts were labeled with [3H]leucine (see "Methods") and then allowed to revert back to rods by transfer to the normal growth medium containing peptone. When the conversion to rods was complete, the PM was isolated and analyzed by SDS-PAGE. The results in Fig. 4A show that pre-BR was not processed to the mature protein. When these cells containing [35S]Met-labeled pre-BR were incubated in the presence of [3H]Leu, (see "Methods"), the PM now isolated had incorporated [3H]leucine into mature BR (Fig. 5B). Pre-BR containing the 35S label again was not processed. The results show that the pre-BR that accumulated in the spheroplasts was not processed, even when the cells had regained their normal morphology and were engaged in synthesis of new mature BR.

Pre-BR in PM Behaves As an Integral Membrane Protein

A possible explanation for the observed lack of processing of the pre-BR is that the precursor exists in an aggregated form and it coalesces with the PM due to its extreme hydrophobic nature. Aggregated proteins that are loosely associated with membranes can be removed by washing the latter with salts, low concentrations of alkali (27), or mild detergents. A preparation of PM containing 35S label in the pre-BR and 3H in BR (see above) was washed with 6 M urea, 6 M guanidine HCl, 100 mM NaOH, and Tween 80. The results in Table I show that prolonged washing with any of these reagents did not result in the loss of the pre-BR (35S label). Furthermore, the experimental conditions did not result in any losses of radioactivity, showing the absence of any solubilization under these conditions.

The Orientation and Conformation of Pre-BR in the PM Is Indistinguishable from That of BR

Proteolysis by Pronase—BR in PM is mostly protected against proteolysis by Pronase, whereas BR precipitated after delipidation undergoes complete hydrolysis to dipeptides and tripeptides. Only the COOH terminus of BR is susceptible to proteolysis by Pronase. In order to determine the conformation and orientation of pre-BR in the PM, the latter was treated with Pronase and analysis by SDS-PAGE. Fig. 5A showed that a small peptide was cleaved. To show that this cleavage occurred from the COOH terminus, Edman degradation of labeled pre-BR after Pronase treatment was carried out and the NH2 terminus was found to be intact. The above results demonstrate that pre-BR folds, like BR, in the PM such that only its COOH terminus is susceptible to proteolysis.

Proteolysis by Chymotrypsin—Cell envelope preparations from *H. halobium* contain BR with the COOH terminus on the inside surface as found in the intact cell. Chymotrypsin cleaves BR into two fragments, C-1 (amino acids 71–248) and C-2 (amino acids 1–71), by cleavage between helices 2 and 3 (18), a region exposed to the outer surface of the cell membrane. If the pre-BR is incorporated into the PM with the same orientation as BR, then proteolysis by chymotrypsin should take place and it should yield the fragment C-1 and another fragment that would be longer than C-2 by 13 amino acids, the precursor sequence.

Cell envelopes containing unlabeled BR and [35S]methionine-labeled pre-BR were treated with hydroxyamine to obtain the apomembrane, and the latter were incubated with chymotrypsin. Cleavage occurred to form C-2 (35S-labeled), C-2 (Coomasie blue staining, not radioactively labeled), and another fragment containing 35S which traveled slower than C-2 on polyacrylamide gels (Fig. 5B). Thus, BR and pre-BR both were cleaved at one identical site by chymotrypsin. In agreement with previous findings (6), when BR and pre-BR in the above *H. halobium* envelopes were treated with chymotrypsin, the digestion of labeled pre-BR into two fragments, C-1 (35S-labeled), and C-2 (35S-labeled), shows that the orientation of pre-BR in the PM is indistinguishable from that of BR.
motrypsin without removal of retinal, very little cleavage occurred.

Proteolysis by Nonspecific Protease—BR in PM sheets is cleaved by a nonspecific S. griseus protease into two fragments, termed S1 and S2. The cleavage occurs between helices 5 and 6, and all the peptide bonds between residues 164 and 168 are equally prone to proteolysis (Fig. 1A). BR in PM is cleaved by the protease only in the presence of Mg2+ at concentration of 10 mM or greater. The interhelical region between helices 5 and 6 in BR is not available for proteolysis. However, when BR is in cell envelopes it is resistant to the S. griseus protease because the above cleavage site is located on the inner surface (See Fig. 1A). The results in Fig. 5C show that pre-BR in cell envelopes is also inert to proteolysis by the protease. However, disruption of the envelopes by sonication results in rapid proteolysis of the precursor to two polypeptides, S'1, which contains the precursor sequence followed by the amino acids 1-163/167, and S2, which contains amino acids 164/168-248 (Fig. 5C). These results demonstrate that in the H. halobium envelopes the conformation and orientation of the interhelical region between helices 5 and 6 are the same in pre-BR and in BR.

Pre-BR Binds Retinal at the Same Site As BR

PM containing 35S-labeled pre-BR was bleached, and the chromophore was regenerated by the addition of [3H]retinal as described under “Methods.” The Schiff's base in the regenerated chromophore was treated with sodium borohydride, either in total darkness or under illumination. The [3H]retinal radioactivity in BR and pre-BR was measured after separation by SDS-PAGE. The results in Table II show that sodium borohydride treatment in the dark resulted in very low incorporation of [3H] label into pre-BR. However, NaBH4 treatment under illumination increased the incorporation of [3H] label 10-fold for both pre-BR and the mature BR. Since reduction of the Schiff's base is known to require illumination (28), the results show that pre-BR and BR behave similarly to borohydride reduction. Therefore, pre-BR also undergoes conformational changes characteristic of the BR photocycle.

35S-labeled pre-BR containing [3H]retinal was reduced as above, and the product was purified by preparative gel electrophoresis and Sephadex LH-60 chromatography in formic acid/ethanol (3:7, v/v). Fragmentation with CNBr and analysis of the CNBr fragments by analytical SDS-PAGE gave the pattern shown in Fig. 6. Thus, the majority of the S label was located in CNBr-6 (amino acids 209-248). Since there is only 1 lysine residue (Lys-216) CNBr-6, retinal is bound to the same lysine residue in pre-BR as in the mature protein. Edman sequence analysis of CNBr-6 containing [3H] label showed 95% recovery of the [3H] label in cycle 7 (Fig. 6, inset). This confirmed the attachment of retinal to lysine 216 (Fig. 1A).

Pre-BR in Membrane, Like BR, Forms Dimers and Trimers on Reaction with Bifunctional Reagents

BR in PM has been shown by Dellweg and Sumper (25) to form dimers and trimers on treatment with the bifunctional cross-linking reagent dimethyl-3,3'-dithiodipropionimidate. If pre-BR forms a similar crystalline lattice, it should also give rise to dimers and trimers. The results in Fig. 7 show that, upon reaction of pre-BR with the bifunctional reagent, cross-linked products identical in mobility with those obtained with BR are formed.

Digestion of Pre-BR with the S. aureus Protease Shows That Its NH2 Terminus Is Exposed to the Outside of the Bilayer

The data presented above show that pre-BR adopts a secondary and tertiary structure similar to that of BR (Fig. 1A). In the latter, some amino acids at the NH2 terminus are
shown as protruding out of the bilayer. It would then be predicted that the additional NH₂-terminal amino acids in pre-BR do not participate in forming an additional helix and thus do not affect the packing arrangement of the seven helices. Proteolytic sensitivity of the precursor sequence in pre-BR was therefore tested.

The S. aureus protease cleaves polypeptide chains at the carboxyl end of glutamate and aspartate residues (26). Because the precursor sequence (Fig. 1 B) contains two glutamate residues, the action of this enzyme on pre-BR was investigated. Pre-BR labeled with [³⁵S]methionine and [³⁵S]leucine (⁰H/⁰S = 9.1 ± 0.15) was incubated in the presence of V8 enzyme for 6 h. Analysis of the products on polyacrylamide gels showed no change in the mobility of pre-BR. However, the ³H/⁰S ratio had increased to 9.92 ± 0.2, suggesting the removal of at least the initiator methionine. The expected ³H/⁰S ratio for the pre-BR polypeptide without the NH₂-terminal methionine is 10.1. Edman degradation of the double-labeled pre-BR gave the following results.

$$\text{Leu} \quad \text{Leu} \quad \text{Leu} \quad \text{Leu} \quad \text{Met} \quad \text{Leu} \quad \text{Leu} \quad \text{Leu} \quad \text{Leu} \quad \text{Leu}$$

The sequence aligns with the protein sequence from residues −4 to 38, thus confirming that the protease had cleaved between amino acid residues 3 and 4 of the precursor.

**Discussion**

The present work has confirmed the earlier conclusion (5, 12) that BR is synthesized as a precursor. The precursor accumulates in H. halobium spheroplasts, and, by amino acid sequencing, we have provided further support for the amino acid sequence of the precursor region. The most significant finding of this work has been that pre-BR is integrated into the PM such that its conformation and orientation are indistinguishable from that of BR. The processing of the precursor sequence is not necessary for the correct insertion and folding of BR within the membrane. The evidence for these conclusions is as follows. Pre-BR copurifies with PM. As expected for an integral membrane protein, it is not removed from PM by washing with alkali (27), salts, and mild detergents. Treatments of selectively labeled pre-BR with Pronase, α-chymotrypsin, and a bacterial protease show that (a) pre-BR, like BR, is mostly shielded from proteolysis and (b) the selective cleavages observed are identical with those previously characterized for BR. Furthermore, the ability of pre-BR to bind retinal at the same site as previously established for BR and the light-dependent reduction of the resulting Schiff’s base show that the retinal-binding pocket has been correctly formed. Finally, the ability to form oligomers in the presence of bifunctional cross-linking reagents suggests that pre-BR is present as trimers in the crystalline lattice of the PM.

It is not known at this time whether upon integration of pre-BR the extra NH₂-terminal amino acids are partially embedded in the membrane to form an additional α-helix or whether they are present at the surface of the membrane. Our experiment with the S. aureus protease showed cleavage at Glu-(−11), the third amino acid from the NH₂-terminal methionine, but no degradation was observed at Glu-4. This result shows that at least a part of the precursor is shielded from proteolysis.

Attempts to demonstrate the in vitro enzymatic processing of the precursor sequence failed. Detergent-solubilized pre-BR was treated with extracts of H. halobium in a variety of media and conditions. In no case was any processing of pre-BR observed. The failure could be due to a number of reasons. 1) In the folded conformation, the precursor region is shielded, and 2) the processing enzyme is unstable. Not having succeeded in isolating the processing enzyme, we can only surmise regarding the possible location of the enzyme in the H. halobium cell. The enzyme could be located on the outside surface of the cytoplasmic membrane, in analogy with the leader peptidase of Escherichia coli characterized by Zwienski et al. (28). The apparent loss of function in the spheroplasts would be consistent with this postulate.

Bacteriorhodopsin belongs to the class of membrane proteins that have the NH₂-terminus out of the cell and have cleavable NH₂-terminal signal sequences. However, the properties of pre-BR now reported, especially its inability to be processed even when the spheroplasts revert to rod-shaped cells, indicate one of two possibilities: either that the processing of the pre-BR is cotranslational or that the NH₂-terminus of the integrated pre-BR somehow becomes inaccessible to the processing enzyme.

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**REFERENCES**


**Fig. 7.** Formation of oligomers with bifunctional reagents. Labeled pre-BR in PM was treated with dimethyl-3,3'-dithiobispropionimidate and analyzed by SDS-PAGE (12% gels).
Bacteriorhodopsin Precursor

Bacteriorhodopsin precursor. Characterization and its integration into the purple membrane.

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