Bacterioopsin-triggered Retinal Biosynthesis Is Inhibited by Bacteriorhodopsin Formation in Halobacterium salinarium*

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Factors regulating retinal biosynthesis in halobacteria are not clearly understood. In halobacteria, events leading to the biosynthesis of bacteriorhodopsin have been proposed to participate in stringent regulation of retinal biosynthesis. The present study describes a novel approach of in vivo introductions of mRNA and membrane proteins via liposome fusion to test their role in cellular metabolism. Both the bacterioopsin-encoding mRNA and the liposome-encapsulated bacterioopsin (apoprotein) are independently introduced in spheroplasts of the purple membrane-negative strain Halobacterium salinarium that initially contain neither bacterioopsin nor retinal. Isoprenoid analyses of these cells indicate that the expression/presence of bacterioopsin triggers retinal biosynthesis from lycopene, and its subsequent binding to opsin generates bacteriorhodopsin. When bacteriorhodopsin and excess retinal were independently introduced into spheroplasts of purple membrane-negative cells, the introduction of bacteriorhodopsin resulted in an accumulation of lycopene, indicating an inhibition of retinal biosynthesis. These results provide direct evidence that the formation of bacterioopsin acts as a trigger for lycopene conversion to \( \beta \)-carotene in retinal biosynthesis. The trigger for this event does not lie with either transcription or translation of the \( bop \) gene. It is clearly associated with the folded and the membrane-integrated state of bacterioopsin. On the other hand, the trigger signaling inhibition of retinal biosynthesis does not lie with the presence of excess retinal but with the correctly folded, retinal-bound form, bacteriorhodopsin.

The study of light-activated retinal proteins (1) provides us with information on how organisms adapt to their environments using light. One such organism that has attracted scientific attention is Halobacterium salinarium. This organism contains three unique retinal proteins that control diverse functions: (a) bacteriorhodopsin involved in light-induced energy transduction (2, 3), (b) sensory rhodopsin controlling light-induced movement (4), and (c) halorhodopsin involved in light-induced chloride transport (5). Retinal acts as a chromophore and is essential for the light-induced activity of these retinal proteins. In the case of bacteriorhodopsin (bR), the apoprotein bacterioopsin (bOp) is attached to all-trans-retinal via the Lys-216 residue to form the chromoprotein bR (6).

In halobacteria, intermediates in the pathway of retinal biosynthesis are well characterized, and its final biosynthetic stages are known to involve the cyclization of lycopene to \( \beta \)-carotene, followed by an oxidation of \( \beta \)-carotene to all-trans-retinal (7). Early studies indicate that retinal biosynthesis in halobacteria may be stringently regulated, and factors involved in such a control remain poorly understood. There are four possible regulatory factors: (a) transcriptional events leading to bacterioopsin mRNA, (b) the translation of \( bop \) mRNA to bacterioopsin protein, (c) all-trans-retinal, and (d) the folded chromoprotein bacteriorhodopsin.

In this study, we report a new approach to understand the regulating factors involved in retinal biosynthesis in halobacteria. In particular, this approach involves the in vivo introduction of mRNA or membrane proteins via liposome fusion into halobacterial spheroplasts. Three potential regulatory elements, \( bop \) mRNA (the transcriptional product of the \( bop \) gene), bOp (the translational product), and bR (the final folded protein), were independently introduced into spheroplasts of purple membrane-negative (Pum\(^-\)) strains that initially contain neither bacterioopsin nor retinal. Subsequent isoprenoid analysis shows that bacterioopsin triggers the biosynthesis of retinal. Furthermore, to determine the regulation leading to inhibition of retinal biosynthesis, both excess retinal and chromoprotein bR were independently introduced into Pum\(^-\) spheroplasts. These results show that although bacterioopsin triggers retinal biosynthesis, it is the formation of bacteriorhodopsin and not the excess of retinal that inhibits its biosynthesis.

**EXPERIMENTAL PROCEDURES**

Materials—Synthetic oligonucleotides were custom synthesized from Genetech Associates (Mumbai, India). Restriction enzymes and other enzymes such as T7 RNA polymerase were purchased from Bangalore Genei (Bangalore, India). Other chemicals were obtained from Sigma. N-NBD-PE and N-Rh-PE were obtained from Molecular Probes. 1,2-Dimyristoyl-glycero-3-phosphocholine, and CHAPS were purchased from Fluka Inc. (Buchs, Switzerland). Lipofectin was purchased from Life Technologies, Inc. Bacto yeast extract and Bacto-tryptone were obtained from Difco. all-trans-Retinal was a kind gift from Dr. A. K. Singh (Chemistry Department, Indian Institute of Technology, Bombay, Mumbai, India).

Pum\(^-\) strains were obtained by screening several colonies of *H. salinarium* S9 on agar plates as orange-colored colonies. These colonies were further characterized using Southern hybridization with a \( bop \) gene-specific probe, as described previously (8). Two strains were found to be of the SD9 type and were characterized as Pum\(^-\), strains in which

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1 The abbreviations used are: bR, bacteriorhodopsin; bOp, bacterioopsin; CHAPS, 3-
(3-cholamidopropyl)dimethylammonio)-1-
propanesulfonic acid; N-NBD-PE, N-7-nitrobenz-2-oxa-1,3-diazol-4-yl-
1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine; N-Rh-PE, rhodamine B 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine; triethylammonium salt; Pum, purple membrane; PAGE, polyacryl-
amide gel electrophoresis; FRET, fluorescence resonance energy transfer.

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the bop gene has been found to be inactivated by the insertion of the IS1 type of transposable element, as described previously (8, 9).

Construction of pGEM-SBOP-L" and pGEM-SBOP-L"—Two genes encoding bacterioopsin with and without leader sequences were synthesized using synthetic oligonucleotides and assembled as shown in Fig. 1. The 5′-region contains the Shine-Dalgarno sequence for Escherichia coli expression (10) and the bop leader sequence, as reported previously (11). The remaining bop sequence was adapted as described by Dunn et al. (12), with the minor modification of replacing the 3′-terminal EcoRI site with HindIII. Both genes were cloned into pGEM(2) vector (Promega, Madison, WI) at EcoRI/HindIII sites.

In Vitro Run Off Transcription—Both plasmids pGEM-SBOP-L" and pGEM-SBOP-L")-were linearized at HindIII sites, and the corresponding bop mRNAs were obtained using in vitro T7-based transcription (13).

Lipofection of Spheroplasts Using bop mRNA—Lipofectin-complexed bop mRNA (Lipofectin::mRNA ratio, 2.5:1) was prepared as described previously (14) and mixed with a suspension of spheroplast cells (3:5 v/v; see below). The mixture was incubated at 37 °C for 45 min.

E. coli Expression of Bacterioopsin—Bacterioopsin samples with and without the leader peptide sequence were isolated and purified after isopropyl-1-thio-galactopyranoside-induced E. coli expression as described previously (13, 15). E. coli-expressed bOp was refolded using halobacterial lipids and all-trans-retinal (13) to obtain bR.

Preparation of bOp- and bR-containing Liposomes—bOp- and bR-containing liposomes were prepared using sonication (16, 17). A chloroform/methanol solution containing 2 mg of egg phosphatidylcholine and 18 mg of acetone-ether-washed halobacterial lipids (18) was dried under N₂ gas, lyophilized, and dispersed into 0.5 ml of 40 mM HEPES-KOH, pH 7.0, and 100 mM KCl. The lipid suspension was then diluted containing liposomes were prepared using sonication (16, 17). A chloroform/methanol solution containing 2 mg of egg phosphatidylcholine and 18 mg of acetone-ether-washed halobacterial lipids (18) was dried under N₂ gas, lyophilized, and dispersed into 0.5 ml of 40 mM HEPES-KOH, pH 7.0, and 100 mM KCl. The lipid suspension was then diluted

Protein Expression and Isolation—After liposome fusions and the appropriate incubations, spheroplast solutions were washed with basal salts containing 0.1 M MgCl₂ and the cells were suspended in growth medium containing peptone (the volume was the same as that used during the original growth). The cells were grown for 12 h, harvested by centrifugation (20,000 × g for 30 min), and washed with 4 M NaCl. For anaerobic growth, cells were grown on 0.5% arginine and in the presence of nitrogen as described previously (21). 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 three times with distilled water (20 ml). Samples were removed and subjected to SDS-PAGE. After the final wash, the pellet was layered on a 15–65% (w/v) sucrose density gradient (25 ml) and centrifuged at 150,000 × g for 16 h (23). The purple band of bR was isolated and
washed four times with distilled water (25 ml) to remove sucrose. Bacteriorhodopsin obtained in this manner was characterized using UV-visible spectroscopy and SDS-PAGE. For electrophoresis, equal amounts of cells were lysed with water, and aliquots were separated by electrophoresis using 15% SDS-PAGE.

**Isolation of Isoprenoids from Spheroplasts**—Isoprenoids were isolated from *H. salinarium* cells as described previously (24). Spheroplasts grown in a 100-ml volume were lysed in 1 ml of water and treated with DNase. Under vigorous stirring, the lysate was added into 9 ml of acetone. After 20 min in the dark, 4 ml of *n*-hexane and 1 ml of water were added. The upper phase containing the pigments was evaporated under a vacuum, and the pigments were diluted in 100 μl of toluene. The sample was applied to an Alumina column (16 g; activity grade II). The elution of β-carotene was carried out by adding 60 ml of 20% diethyl ether in *n*-hexane, and the elution of retinal was carried out by a further addition of 140 ml of the same solvent mixture. The elution of lycopene was carried out with 40 ml of diethyl ether. The amount of each compound obtained was calculated spectrophotometrically (25). The values are expressed on a protein basis (26) as micrograms/gram of cellular proteins.

**RESULTS**

**Overall Strategy**—A threefold experimental strategy has been used in this study: (a) to see whether the presence of bOp acts as a trigger for retinal biosynthesis, *bop* mRNA was introduced using lipofection and subsequently translated into a *Pum* strain; (b) to see whether exogenously added bOp triggers retinal biosynthesis, the apoprotein bOp was introduced into spheroplasts via liposome fusion; and (c) to distinguish between apoprotein- and chromoprotein (*retinal-containing protein*)-mediated control, bR was introduced into spheroplasts via proteoliposome fusion. Cells were lysed to assay the presence of bR and to determine the change in isoprenoid composition (the amount of lycopene, β-carotene, and retinal). Lycopene is known to be a precursor of retinal (27), and its presence has been used to track retinal biosynthesis in halobacteria (7).

**Spheroplasts of Pum Strains Do Not Produce bR or bOp**—Spheroplasts were prepared according to methods described previously (20, 28). The viability of spheroplasts was determined by titrating them on spheroplast regeneration plates as described previously (29, 30), and it was found that spheroplasts were viable. Fig. 2A shows SDS-PAGE analysis of partially purified membranes from spheroplasts. The samples subjected to SDS-PAGE were aliquoted before the sucrose density gradient step (23). Fig. 2A (lane 2) shows the clear absence of bOp in spheroplasts of *Pum* origin. The absorption spectrum of a sample subjected to SDS-PAGE also shows a lack of the characteristic absorption peak corresponding to bR at 560 nm (Fig. 2A, inset), suggesting that spheroplasts of the *Pum* strain do not produce bR or bOp.

**Liposome-mediated Introduction of bop mRNA into Spheroplasts of Pum Strains**—**Results in the Expression of bOp and the Subsequent Formation of bR**—Fig. 2A shows that when Lipofectin-mediated introduction of *bop* mRNA was carried out in *Pum* spheroplasts, bacteriorhodopsin is produced in the correctly folded form, as seen from SDS-PAGE (lane 4) and the characteristic 560 nm absorption peak (13, 31) (Fig. 2A, inset). Sucrose density gradient ultracentrifugation shows that bR expression leads to its assembly as a purple membrane lattice. This indicates that exogenously-added *bop* mRNA is translated efficiently in spheroplasts of *Pum* cells. In a control experiment in which only Lipofectin lacking mRNA was used, no bOp production was observed (Fig. 2A, lane 3), thus indicating that the transfection protocol did not produce a latent/proviral integration gene. Another control experiment in which *bop* mRNA was lipofected into *Pum* cells and grown in the presence of N2 gas (see below) showed that bR formation does not take place. This is due to the inhibition of the oxidation of β-carotene to retinal. The newly isolated bR showed characteristic light-dark adaptation and proton pumping activity (data not shown). These results indicate that bOp translated from *bop* mRNA is integrated into the membranes of halobacterial cells and binds to intrinsic retinal to form a functional bacteriorhodopsin.

It has been shown that spheroplasts are incapable of processing immature bR, and only when they are returned to normal cells can they process bR to cleave off the leader sequence (20). Fig. 2B shows the presence of unprocessed bR in spheroplasts of *Pum* cells that were lipofected with *bop* mRNA containing the leader sequence and where the spheroplasts had yet to revert to normal cells (lane 3). Lane 4 shows the presence of both unprocessed bR and processed bR in spheroplasts that were allowed to convert to normal cells. When mRNA lacking the leader sequence was used in lipofection, no detectable bOp expression was found (lane 2). This observation is in agreement with previously reported works (11, 32) in which it has been indicated that the leader sequence of *bop* mRNA is essential for binding to the ribosomal binding site in halobacteria. The hairpin structure formed by *bop* mRNA may also add to the intracellular stability of the exogenously introduced mRNA (11, 32).

**bOp and bR Can Be Introduced in Vivo into Membranes of Spheroplasts of H. salinarium**—The fusion of proteoliposomes containing bOp and bR was done with spheroplasts of the *Pum* strain of *H. salinarium*. The isolation and purification of protein show that the exogenously introduced apoprotein bacteriorhodopsin forms the chromoprotein bacteriorhodopsin and is incorporated into the membranes of *H. salinarium* (Fig. 2A, lane 5). After reconstitution, the isolated chromoprotein exhibited normal proton pumping activity, indicating that the pro-
tein is functional (data not shown).

The liposome fusion process was monitored using FRET. Fluorescent donor-labeled lipids and acceptor-labeled lipids (N-NBD-PE and N-Rh-PE; see “Experimental Procedures”) were incorporated into liposomes and fused with spheroplasts. In the case of proteoliposomes containing bR, the fluorescence of N-NBD-PE is quenched by bR due to its strong absorption at 560 nm. FRET analysis was performed, and the results indicated that over a period of 1 h, the fluorescence intensity increased as the donor- and acceptor-labeled lipid/bR molecules move away from each other within the membrane as a result of fusion. As a result, a decrease in the quenching of fluorescence occurs as the distance between the label and the protein increases as a function of time (Fig. 3). These results show that liposome fusion has taken place with the spheroplast membranes of *H. salinarium*. Fusion efficiency is estimated (33) and is found to be in the range of 50–60%. In control experiments, neither spheroplasts nor liposomes alone showed any appreciable fluorescence change over a 1-h period. Spheroplasts alone do not show any detectable emission at 530 nm (λex, 465 nm).

**Lycopene Cycling and Subsequent Retinal Biosynthesis Is Induced by the Presence of Membrane-bound bOp—Pum**

-regenerated cells. Pigment analysis of these cells (Table I, column 5) showed lycopene accumulation and hence the inhibition of the conversion of lycopene to β-carotene by bR. The exogenously introduced bR was isolated and found to be functionally unaltered when checked for proton pumping (data not shown).

**Exogenously added Retinal Does Not Inhibit Lycopene Cyclization**—It has been established that the conversion of β-carotene to retinal is a spontaneous and oxygen-dependent process and is inhibited under anaerobic conditions (7). Under anaerobic conditions, β-carotene should accumulate, and retinal should be absent. When spheroplasts of Pum− cells were lipofected with bop mRNA and grown under anaerobic conditions, although bOp-induced lycopene-to-β-carotene conversion is seen (Table I, column 6), β-carotene-to-retinal conversion remains inhibited. When excess retinal is introduced in Pum− cells lipofected with bop mRNA under anaerobic conditions, excess retinal does not seem to inhibit lycopene-to-β-carotene conversion (Table I, column 7), as evidenced by the increased β-carotene content in contrast to the data shown in Table I, column 8. It should be noted that if bR is introduced under similar anaerobic conditions in the presence of excess retinal, no β-carotene is formed (Table I, column 9), indicating that it is bR and not the excess retinal that is inhibiting the lycopene-to-β-carotene conversion.

**DISCUSSION**

Purple membrane, a differentiated domain of the plasma membrane, contains only one protein species, bacterioopsin, which is complexed with retinal in a 1:1 stoichiometry. The biogenesis of purple membrane is inducible by limiting the oxygen supply (34) which turns on the synthesis of both bacterioopsin and retinal (35). In contrast, most of the lipid molecules necessary for purple membrane formation are synthesized long before the start of bacterioopsin and retinal synthesis, i.e. lipids are taken from the pool of the cell membrane (24, 35).

Most of the retinal present in halobacteria is associated with bacterioopsin; hence, one observes a nearly stoichiometric relationship between bacterioopsin and retinal content. This fact suggests the existence of a highly efficient regulation mechanism coordinating the bacterioopsin and retinal synthetic pathways. In a classic piece of work by Sumper and Herrmann (24), it has been found that whereas bOp may be the trigger for retinal biosynthesis from its precursors, it is unclear which of the two (excess retinal or bacteriorhodopsin in the membrane) generates the feedback signal to stop retinal biosynthesis. From the existing data, the following questions emerge on events that trigger retinal biosynthesis (Fig. 4):

(a) Which of the two steps (lycopene-to-β-carotene conversion or β-carotene-to-retinal conversion) is triggered by bacterioopsin?

(b) Which of the two events (bop transcription or bop translation) acts as a trigger of retinal biosynthesis?

(c) If, instead of possibility b, protein product bOp is the trigger, which form of the protein (unfolded nascent polypeptide or folded polypeptide) acts as a trigger of retinal biosynthesis?

The results of the present study aimed at understanding the factors triggering retinal biosynthesis show that both endogenously expressed bOp and exogenously introduced bOp are converted into the retinal-bound form, bacteriorhodopsin. A concomitant decrease in the lycopene content and the formation of retinal suggests that the presence of the apoprotein bOp has triggered the conversion of lycopene to β-carotene and hence the formation of retinal. Furthermore, our results show that this trigger operates at the lycopene-to-β-carotene conversion and not at the β-carotene-to-retinal conversion. It should be noted that nicotine binding and oxygen dependence studies show that the β-carotene-to-retinal conversion is spontaneous (7, 35).

The fact that bop mRNA as well as bOp triggers the lycop-
Regulation of Retinal Biosynthesis in Halobacteria

TABLE I

| Lycopene | Trace | 1700 ± 30 | Trace | 1600 | 1700 ± 30 | 1500 ± 30 | 1800 ± 100 | 1380 ± 180 |
|-----------------|----------|-------------|----------|-----------------|-------------|-------------|-------------|-------------|
| β-Carotene      | 560 ± 30 | 0           | 480 ± 40 | 450 ± 34         | Trace       | 1430 ± 30   | 1480 ± 10   | Trace       |
| Retinal          | 44 ± 3(54) | 0           | 40 ± 8(60) | 24 ± 5(27)         | 25 ± 5(27) | Trace       | Yes         | No          |
| bR formation    | Yes      | No          | Yes      | Yes             | Trace       | Yes         | No          | No          |

* Values of retinal content expected from the isolated bacteriorhodopsin content.
A Value of retinal expected from the averaged 50% fusion efficiency of bOp- or bR-containing proteoliposome fusion.

a. Which of the two (excess retinal or bR) acts as a trigger for inhibition?

At what stage does the feedback signal operate, e.g., at the lycopene-to-β-carotene conversion or at the β-carotene-to-retinal conversion?

These questions are addressed by the in vivo introduction of both bR and excess retinal. When bR is introduced into Pum−spheroplasts, lycopene accumulation occurs, suggesting that the presence of bR triggers the inhibition of retinal biosynthesis. In contrast, when excess retinal is introduced, no lycopene accumulation/inhibition occurs, indicating that the trigger signaling inhibition does not lie within the presence of excess retinal. Also, when the Pum− strain containing excess retinal was allowed to grow anaerobically in the presence of bOp, it did not inhibit bOp-induced lycopene-to-β-carotene conversion. Thus, we conclude that it is the presence of bR that inhibits and then regulates the lycopene-to-β-carotene conversion and imparts stringency to retinal biosynthesis.

Fig. 4. Schematic representation of retinal biosynthesis and its regulation by bacterioopsin and bacteriorhodopsin. Dotted lines indicate the effect with which bR or bOp acts in an inhibitory (−ve) or stimulatory (+ve) manner on the biosynthetic pathway.

Pum−spheroplasts

**Isopentenyl-PP**

**Phytoene**

**Phytofluene**

**α-Carotene**

**Neuropigrene**

**Lycopene**

**Bacteriorhodopsin**

**β-Carotene**

**All-trans retinal**

**Bacterioopsin**

pene-to-β-carotene conversion indicates that the trigger does not lie within the events associated with transcription or translation of the bop gene. The next question is which form of bOp (the totally unfolded form or the partially folded form) triggers retinal biosynthesis. In the present study, bOp has been introduced as the partially folded or membrane-integrated form. Due to the extreme hydrophobicity of bOp (16) and the membrane-associated nature of proteoliposomes, we rule out the possibility of direct introduction of bOp in the cytoplasm of Pum−spheroplasts. Furthermore, the study of Driessen et al. (16) and our regeneration experiments on proteoliposomes suggest that bOp was introduced only in the correctly folded form. Thus, we conclude that bOp-mediated retinal formation is not associated with the unfolded nascent polypeptide present in the cytoplasm. Instead, the trigger is clearly associated with the folded and the membrane-integrated form of bOp.

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Regulation of Retinal Biosynthesis in Halobacteria

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