brp and blh Are Required for Synthesis of the Retinal Cofactor of Bacteriorhodopsin in Halobacterium salinarum*

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Bacteriorhodopsin, the light-driven proton pump of Halobacterium salinarum, consists of the membrane apoprotein bacterioopsin and a covalently bound retinal cofactor. The mechanism by which retinal is synthesized and bound to bacterioopsin in vivo is unknown. As a step toward identifying cellular factors involved in this process, we constructed an in-frame deletion of brp, a gene implicated in bacteriorhodopsin biogenesis. In the Δbrp strain, bacteriorhodopsin levels are decreased ~4.0-fold compared with wild type, whereas bacterioopsin levels are normal. The probable precursor of retinal, β-carotene, is increased ~3.8-fold, whereas retinal is decreased by ~3.7-fold. These results suggest that brp is involved in retinal synthesis. Additional cellular factors may substitute for brp function in the Δbrp strain because retinal production is not abolished. The in-frame deletion of blh, a brp paralog identified by analysis of the Halobacterium sp. NRC-1 genome, reduced bacteriorhodopsin accumulation on solid medium but not in liquid. However, deletion of both brp and blh abolished bacteriorhodopsin and retinal production in liquid medium, again without affecting bacterioopsin accumulation. The level of β-carotene increased ~5.3-fold. The simplest interpretation of these results is that brp and blh encode similar proteins that catalyze or regulate the conversion of β-carotene to retinal.

Rhodopsins are integral membrane proteins containing seven transmembrane α-helices and a covalently bound molecule of retinal. Two distinct rhodopsin families are known: the visual rhodopsins, which bind 11-cis retinal or related compounds and function as photoreceptors in vertebrates (1) and invertebrates (2), and the archaeal rhodopsins, which bind all-trans-retinal and function as light-driven ion pumps and phototaxis receptors in archaea (3). Archaeal rhodopsin orthologs have been found recently in bacteria (4) and fungi (5), suggesting that retinal-based pigments are of widespread significance. Despite their importance, the biogenesis of these molecules is not fully understood. In particular, relatively little is known about how retinal is assembled with the opsin apoprotein in vivo. Thus, a goal in elucidating rhodopsin biogenesis is to identify the cellular factors that mediate the biosynthesis or uptake of retinal, the transport of retinal in the cell, and the binding of retinal to the corresponding opsin.

To this end, we have studied the biogenesis of bacteriorhodopsin (BR), a light-driven proton pump in the archaeon Halobacterium salinarum. BR consists of the membrane protein bacteriopsin (BO) and all-trans-retinal. Under microaerobic conditions, BR is induced ~50-fold (6) and forms a two-dimensional crystal known as the purple membrane. This system has served as a model for studying key steps in membrane protein biogenesis, including protein insertion into the membrane (7, 8) and the assembly of protein-lipid complexes (9, 10). H. salinarum is genetically tractable, and the genome sequence of a closely related organism, Halobacterium sp. NRC-1, has been determined (11). Thus, the prospect of identifying the cellular factors that mediate retinal assembly and other steps in BR biogenesis in H. salinarum is excellent.

Retinal is synthesized de novo in H. salinarum (12) and eventually binds BO to form BR. A pathway for retinal biosynthesis has been proposed from studies of cell-free preparations and by comparison with other carotenoid biosynthetic pathways (12). Intermediates in the pathway from the universal $C_{40}$-carotenoid precursor phytene to β-carotene have been identified (12). β-Carotene is thought to be the immediate precursor of retinal, although there is no direct evidence for its conversion to retinal in H. salinarum. Furthermore, the cellular factors that catalyze this conversion are unknown. The addition of retinal to BO to form BR has long been supposed to occur without the participation of cellular factors because BR can be regenerated from retinal and purified BO in vitro (13, 14). However, cellular factors may be required to prevent the photooxidation or photoisomerization of the cofactor during its transport or binding to BO. These functions may be mediated by a retinal-specific chaperone or by a multifunctional enzyme that converts β-carotene to retinal and transports or binds retinal to BO.

As a first step to identify cellular factors that mediate these processes, we chose to study the brp gene, which encodes a putative membrane protein (Brp) implicated in BR biogenesis (15–18). The brp gene is part of a gene cluster (Fig. 1) that includes bop, which encodes BO; bat, which encodes a transacting factor (Bat) that contains a region homologous to the PAS domain of the oxygen sensor NifL (19, 20) and activates bop expression under microaerobic conditions (21, 22); and blp, a gene of unknown function (23). Insertions in brp greatly decrease BR and bop mRNA levels (15–18). This result can be
interpreted to imply that Brp modulates bop expression (18, 21). However, it has also been recognized that the effect of brp insertions may be attributable to an indirect effect on bop expression (18, 21). The bop gene is immediately downstream of brp, and the termination and initiation codons of the two genes overlap (Fig. 1). Northern blot analysis of brp and bop mRNAs was interpreted as evidence for separate transcripts, although cotranscription of the two genes was not excluded (21). Thus, brp and bop may constitute an operon, and insertions in brp may reduce bop expression indirectly by a polar effect on bop transcription. In this case, Brp may play a role in BR biogenesis other than regulating bop expression, such as the biosynthesis, transport, or binding of the retinal cofactor of BR.

To examine the role of Brp, we created an in-frame deletion of brp using a recently developed ura3-based gene knockout strategy (24). Analysis of BR, BO, and carotenoid accumulation in the deletion strain suggests that Brp is essential for the production of BR but not BO. Instead, bop appears to be required for the synthesis of retinal from β-carotene. Parallel studies with blh, a paralog of brp identified from the Halobacterium sp. NRC-1 genome sequence (11), indicate that the blh gene product partially substitutes for Brp. The implications of these findings for BR biogenesis and retinal biosynthesis in H. salinarum are discussed.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from Operon (Alameda, CA). Taq polymerase was obtained from Promega (Madison, WI), and restriction endonucleases and ligase were obtained from New England Biolabs (Beverly, MA). All other reagents were obtained from Sigma.

Plasmid Construction—Plasmids were propagated in the Escherichia coli strain DH5α except where noted. A two-step polymerase chain reaction (PCR) was performed to construct the ∆brp plasmid containing DNA homologous to brp with an in-frame deletion of codons 33–308 and an 18-bp insertion at the deletion site. In the first step, reactions were carried out with the primer pairs GTACCGAGCCATCTCGAGACT, CTGCTCGATCTCGATCTCACCG-GATCCCATGCCTACTTCAGTAC and GCCCATGATGTTCATCGACT, respectively. Sequencing reactions were analyzed with a 373XL automated DNA sequencer (Applied Biosystems, Inc.) at the University of Washington Biotechnology Center.

Induction and Preparation of Cell Lysates—To induce BR synthesis, 120 ml of peptone medium (27) in a 215 ml Erlenmeyer flask were inoculated with 1.2 ml of saturated H. salinarum culture and grown in an orbital shaker at 40 °C with shaking at 250 rpm for 96–100 h. Where noted, 30 μl of 10% retnal in isopropl alcohol were added at 14, 24, 38, 48, and 72 h after culturing. At an Od600 of 0.70–0.85, the cultures were harvested by centrifuging at 8000 × g for 30 min at 4 °C. The cell pellet was resuspended in 100 ml of basal salts and centrifuged again at 8000 × g for 30 min, followed by a brief spin to remove all traces of the supernatant. The cell lysates were lyophilized and resuspended in 3.5 ml of 4% SDS, 0.5% phenylmethylsulfonyl fluoride, and 0.025% sodium azide in water and shaken for ~1 h at room temperature. Samples were purged with nitrogen gas to minimize carotenoid oxidation.

Quantification of BR and BO—To assess BR levels, cell lysates were diluted 1:4 in 30 mM sodium phosphate buffer (pH 6.9) containing 0.1% sodium azide and scanned in a Perkin-Elmer α2 spectrophotometer. The BO/BR ratios were determined via their characteristic light-dark difference spectrum. BO was quantified by a standard curve described (6) with a standard curve obtained with purified BR added to a lysate from the ∆brp strain MPK412 (24). The BR levels were expressed as a percentage of total cell protein as measured by the BCA assay (Pierce). The BO levels were determined by immunoblotting with BR-114 monoclonal antibody generously provided by Dr. H. G. Kjorvaeren. The blots were subsequently incubated with fluorescein-conjugated α-mouse IgG secondary antibody (Amersham Pharmacia Biotech), and the BO levels were quantified on a Hitachi FMBIOII Multi-View Fluororimeter. Purified BR was used to generate a standard curve.

Extraction and Characterization of Carotenoids—Total carotenoid was extracted from cell lysates as described (28). Lysates were illuminated with 440–460 nm light prior to extraction to convert all retinal to the all-trans isomer. The extracts were evaporated to dryness under nitrogen gas. To identify the major carotenoid in ∆brp strains, the extracts were fractionated by HPLC on an HPLX solvent delivery system (Rainin Instrument Co., Inc., Emeryville, CA) coupled with a reverse phase Ecosphere C18 (250 × 4.6 mm, 5 μm particle size) (Alltech Associates, Inc., Deerfield, IL) and an Alltech Ecosphere C18 5 μm guard column. The mobile phase was a gradient of 100% solvent A (0.05% methanol, 0.01% AcOH) to 100% solvent B (95% solvent A, 5% methanol) over 30 min at a flow rate of 1 ml/min. The solvent change over a 25-min sample run was programmed as follows: elution with 100% solvent A, 3 min; gradient to 32% solvent B, 6 min; isocratic elution with 32% solvent B, 11 min; gradient to 100% solvent A, 1 min; and re-equilibration with 100% solvent A, 4 min. The eluate was monitored at 474 nm with a Dynamax UV-1 variable wavelength UV/visible absorbance detector (Rainin In
An Insertion in brp Eliminates BO Expression—Earlier studies of brp function relied on spontaneous insertions in brp (Fig. 1). Because insertions in brp might reduce brp expression indirectly through a polar effect on bat, we reexamined the role of brp in BR biogenesis. As a first step, we confirmed the phenotype of brp insertions by isolating a spontaneous mutant from a laboratory strain of H. salinarum, MPK1 (6). Unlike MPK1, which forms purple colonies, the mutant strain yielded pale yellow colonies. PCR, Southern blot, and DNA sequence analysis of the mutant strain (data not shown) revealed the presence of the insertion element ISH27 (16) at the third nucleotide position of codon 177 in the open reading frame, which encodes a polypeptide of 359 amino acids. After incubation under lights, Δbrp cell lysates revealed three prominent absorbance maxima at 474 nm and 449 and 451 nm, respectively (data not shown). The slight difference in the ratio of Δbrp to BR (28). The extracts were fractionated with HPLC to quantify β-carotene and decreases retinal levels. In addition to the reduced peak at 570 nm, the UV/visible spectra of Δbrp cell lysates revealed three prominent peaks between 400 and 500 nm that were absent from the wild type (Fig. 3, solid and dotted lines). This suggested that Δbrp had higher levels of β-carotene, which is thought to be the precursor of retinal in H. salinarum (12). To test this possibility, carotenoid was extracted from the wild-type and Δbrp cell lysates using a method that recovers >80% of the retinal bound to BR (28). The extracts were fractionated with HPLC to quantify β-carotene and retinal (Fig. 5). The species eluting at 15 min comigrated with commercial β-carotene and was present at ~3.8-fold higher levels in the Δbrp strain than in the wild type (Fig. 4, samples 1 and 2, and Fig. 5, traces 1 and 2). Independent experiments were carried out to confirm that the major carotenoid from the Δbrp strain is β-carotene. Under different HPLC conditions (see “Experimental Procedures”), the Δbrp extract and commercial β-carotene yielded both a prominent species absorbing at 474 nm that eluted at 17.5 min and a minor species that eluted at 17.8 min, presumably attributable to cis-isomers of β-carotene (data not shown). The HPLC peak fractions obtained from the Δbrp extract and commercial β-carotene had mass ion values of 536.441 and 536.437 Da and similar UV/visible spectra with absorption maxima at 449 and 451 nm, respectively (data not shown). The slight difference in absorption maximum may be attributable to a difference in the ratio of β-carotene isomers (29). Thus, the major carotenoid that accumulates in the Δbrp strain is β-carotene.
Role of brp and blh in Bacteriorhodopsin Biogenesis

The ~3.8-fold increase in β-carotene accumulation in the Δbrp strain was accompanied by a corresponding ~3.7-fold decrease in retinal accumulation (Fig. 4, samples 1 and 2, and Fig. 5, traces 1 and 2). The simplest interpretation of these results is that Brp catalyzes or regulates the conversion of β-carotene to retinal.

Δbrp Is Complemented by an Intact Copy of brp—A complementation strain was constructed to confirm that the Δbrp phenotype is caused by the loss of brp and not by a reduction in bat expression or a second-site mutation in an unknown gene. The brp open reading frame, flanked by 186 bp of the upstream sequence to allow normal expression of the gene, was integrated at the ura3 locus. The resulting complementation strain, Δbrp ura3::brp, yielded purple colonies identical to the wild type. When this strain was grown under BR induction conditions, BR, β-carotene, and retinal levels were restored to the wild-type levels (Fig. 3, dashed line, Fig. 4, sample 3, and Fig. 5, trace 3). These results confirm that the Δbrp phenotype is caused solely by the loss of brp and that the in-frame deletion of brp has no detectable effect on bat expression.

A brp Paralog May Function Similarly to brp—The Δbrp mutation reduced BR levels by ~4.0-fold but did not completely eliminate BR synthesis, raising the possibility that other factors partially substitute for brp in BR biogenesis. To examine this possibility, the Halobacterium sp. NRC-1 genome sequence (11) was searched for genes encoding proteins homologous to Brp, and a single gene, blh, was identified. The blh open reading frame begins with a GUG start codon and encodes a putative 345-amino acid integral membrane protein (Blh) with 28% identity to Brp over its entire length. (blh was initially predicted to encode a 284-amino acid protein (11). However, our analysis suggests that the protein may be 61 amino acids longer at the N terminus based on GC bias in the third position of codons and a better match of the length and predicted topology of the blh and brp gene products.)

To determine whether Blh plays a role in BR biogenesis, an in-frame deletion of blh was constructed in the wild-type and Δbrp backgrounds. Codons 97–300 of the blh open reading frame were deleted. The Δblh colonies had a dark orange color similar to the Δbrp colonies, but the ΔbrpΔblh colonies had a yellow color, suggesting an accumulation of β-carotene without the expression of BR. When grown microaerobically in liquid media to induce BR expression, the Δblh strain had wild-type levels of BR, β-carotene, and retinal (Fig. 4, sample 4, and Fig. 6, dashed line). Given that Δblh colonies were clearly altered, the lack of an observable effect under liquid growth conditions in the dark is surprising but suggests that blh function or expression is sensitive to growth conditions. Significantly, in the ΔbrpΔblh strain, no BR or retinal was detected, and β-carotene levels were ~5.3-fold higher than wild type (Fig. 4, sample 5, and Fig. 6, solid line). The BO levels were normal in both Δblh and ΔbrpΔblh (Fig. 4, samples 4 and 5, and Fig. 6, inset). These results suggest that Blh acts similarly to Brp in converting β-carotene to retinal.

Addition of Retinal in Vivo Restores BR Accumulation in Δbrp and ΔbrpΔblh Strains—One model for Brp and Blh func-
tion is that they aid BO folding to permit retinal binding. To test this possibility, retinal was added periodically to cultures of the Δbrp and ΔbrpΔblh strains during growth under conditions to induce BR. The addition of all-trans-retinal restored BR accumulation to the wild-type levels (Fig. 7), confirming that the BO produced by these strains is competent to bind retinal and suggesting that Brp and Blh are not required for the correct folding of BO.

**DISCUSSION**

We have identified two related *H. salinarum* genes, brp and blh, that are required for BR biogenesis. The in-frame deletion of *brp* alone results in decreased BR and retinal levels and a corresponding increase in β-carotene levels. These effects are enhanced by the deletion of both *brp* and *blh*. The deletion of these genes has no significant effect on BO levels. These results indicate that *brp* and *blh* are not involved in regulating *bop* gene expression. Instead, the genes are needed for the synthesis of the retinal cofactor of BR or for its transport or binding to BO.

The simplest model of *brp* and *blh* function is that they encode the proteins that catalyze or regulate the catalysis of the conversion of β-carotene to retinal. The concomitant decrease in retinal and increase in β-carotene levels in the Δbrp strain strongly support this model. Furthermore, the only defect in strains lacking *brp* and *blh* is the inability to synthesize retinal, because exogenous retinal restores BR levels. However, Brp and Blh have no obvious primary structural features that indicate they catalyze the conversion of β-carotene to retinal. Significantly, Brp and Blh are unrelated to the recently described 15,15′-β-carotene dioxygenase of *Drosophila melanogaster* (30), a soluble protein that catalyzes the oxidative cleavage of β-carotene to two molecules of retinal. Because we have been unable to find orthologs of this enzyme in the *Halobacterium* sp. NRC-1 proteome, Brp and Blh may be part of a novel retinal biosynthetic pathway unique to halarchaea.

An alternative model is that Brp and Blh are involved in the transport of retinal in the cell or the binding of retinal to BO. If the proteins were involved exclusively in transport or binding, at least low levels of retinal would be expected to accumulate in the ΔbrpΔblh strain. The failure to detect retinal in this strain argues against a role of the proteins in retinal transport or binding. However, we cannot exclude the possibility that the proteins are multifunctional enzymes that catalyze the conversion of β-carotene to retinal and also mediate retinal transport and binding.

Another model is that Brp and Blh encode proteins that regulate the expression of enzymes that convert β-carotene to retinal. Although the proteins lack features typical of transcriptional regulators, they may interact with transcriptional regulators to modulate transcription. This type of regulation was suggested previously in a model whereby the *brp* gene product acts as a light sensor (31) and activates Bat to modulate *bop* transcription. We showed that *brp* has important functions without light because the differences in BR and carotenoid levels between wild-type and deletion strains were observed in cultures grown in the dark. Moreover, Brp does not appear to regulate *bop* transcription because BO levels were normal in the Δbrp strain grown either in the dark or in the light (Fig. 4). Thus, Brp is unlikely to be a light-sensing regulator of Bat. Nevertheless, Brp may modulate the activity of Bat at genes other than *bop*, such as those that are required for retinal metabolism.

Our results support the pathway of retinal synthesis in *H. salinarum* proposed previously (12). This pathway was based on the *in vitro* reconstitution of β-carotene formation from mevalonate (32) and on the accumulation of C_{15}-isoprenoid intermediates in colorless mutant strains that lacked β-carotene and retinal (33). However, direct biochemical or genetic evidence for the conversion of β-carotene to retinal has not been obtained. In our experiments, we have shown that the deletion of a single gene (*brp*) simultaneously results in decreased retinal accumulation and increased β-carotene accumulation. When *brp* and *blh* are both deleted, β-carotene accumulation increases further and no retinal is detectable. Thus, β-carotene is likely to be the precursor to retinal in *H. salinarum* and is not converted spontaneously to retinal.

Brp and Blh appear to have redundant functions. The redundancy may be needed to allow retinal production under both aerobic and anaerobic growth conditions. Of the four rhodopsins produced by *H. salinarum*, three are induced microaerobically (BR, halorhodopsin, and sensory rhodopsin I), whereas the fourth (sensory rhodopsin II) is suppressed under these conditions (34). The immunoblotting of cell lysates with an antibody directed against epitope-tagged Brp indicates that Brp is present only in cells grown microaerobically (3). This finding is consistent with a model in which Brp is induced microaerobically to provide the retinal needed for the formation of BR, halorhodopsin, and sensory rhodopsin I, and Blh is expressed aerobically to provide retinal to sensory rhodopsin II.

The results presented here have implications for the regulation of BR biogenesis. BR biogenesis is regulated partly by *bat*, which is required for *bop* and *brp* expression as shown by the virtual absence of *bop* and *brp* mRNAs in *bat* deletion or inser- 

Two factors identified in this study play a key role in the synthesis of the retinal cofactor that is essential for the biogenesis of BR in *H. salinarum*. Further studies are needed to confirm that Brp and Blh catalyze the conversion of β-carotene to retinal and to test whether these proteins play a role in transporting retinal or in binding retinal to BO. It will also be

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2 R. F. Peck and M. P. Krebs, unpublished data.
3 R. F. Peck and M. P. Krebs, unpublished data.
important to determine whether other cellular factors participate in these processes. Such factors may be identified by using the *Halobacterium* sp. NRC-1 genome sequence (11) and the ura3-based reverse genetics approach as we have demonstrated for *brp* and *blh*.

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