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Biochemistry

Expression of the archaebacterial bacterio-opsin gene with and without signal sequences in *Escherichia coli*: The expressed proteins are located in the membrane but bind retinal poorly*

(bacteriorhodopsin/heterologous expression/membrane localization/protein folding/signal peptide cleavage)

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ABSTRACT **In a further effort to obtain functional expression of the bacterio-opsin gene (*bop*) in *Escherichia coli*, the *bop* gene with *E. coli* signal sequences as well as the *bop* gene with the native presequence were expressed in *E. coli*. The location of the expressed products in the *E. coli* cell and their processing and folding to a structure that binds retinal as in *Halobacterium halobium* were investigated. All the expressed proteins were in the membrane. The proteins were largely unprocessed, and they were distributed between the outer and the inner membrane. The processed fractions, which were minor, were exclusively in the inner membrane. The processed proteins bound exogeneously added all-*trans*-retinal but only partially, indicating that these proteins were present in at least two folded states.**

Bacteriorhodopsin (bR), a transmembrane protein in the purple membrane of *Halobacterium halobium*, serves as a light-driven proton pump. It contains a single polypeptide chain of 248 amino acids with seven putative helical segments (Fig. 1) and a molecule of all-*trans*-retinal as the chromophore. The latter is linked to the ε-amino group of Lys-216 (1, 2) as a Schliff base. The gene for bacterio-opsin (*bop*) in *H. halobium* contains a presequence of 13 amino acids at the N terminus (3). For structure–function studies of bR, we have expressed the *bop* gene and its mutants in *Escherichia coli* after removal of the presequence and the addition of an N-terminal methionine codon (4) (Fig. 1). The expressed proteins, which are isolated in denatured form, renature and regenerate bR-like chromophores with exogeneous retinal in detergent/lipid mixtures. To our knowledge the location and state of the expressed bacterio-opsin (bO) *in vivo* in *E. coli* have not been studied so far. If bO expressed in *E. coli* were to exist in correctly folded form in the cytoplasmic membrane and would bind all-*trans*-retinal to regenerate functional bR, a light-dependent proton pump would thus become available for biophysical studies in *E. coli*. In investigating this possibility, we have asked the following questions. (*i*) Where is the expressed bO located in *E. coli*? (*ii*) Does it insert in the membrane? If so, is it in the cytoplasmic membrane only or is it in both the cytoplasmic and the outer membrane? (*iii*) What is the influence of the *E. coli* signal sequences on the location of bO? Are the signal sequences processed? (*iv*) Does bO or its fusions with the signal sequences fold in *E. coli* as in *H. halobium* so as to regenerate the chromophore with all-*trans*-retinal and carry out light-dependent proton translocation? As we report below, bO and the related proteins in *E. coli* indeed are in the membrane, but they are distributed between the inner and outer membranes. The proteins containing the added *E. coli* signal sequences remain largely unprocessed and are distributed between the inner and the outer membrane. The fractions of the proteins that are processed are located exclusively in the inner membrane. These fractions bind retinal but only partially. Finally, we studied the possible effect of the presequence in the native *bop* gene in *H. halobium* on the expression and processing in *E. coli*. Extensive degradation of the expressed protein was observed, the presequence was not cleaved, and the protein did not fold so as to bind retinal.

MATERIALS AND METHODS

Bacterial Strain and Expression System. A T7 RNA polymerase-dependent expression system (5) was used. The *E. coli* strain used was W3110 (F^-, rk^-, mk^+)$lacI^q$ harboring two plasmids, pACT7 and pT7BO4. Plasmid pACT7 carries a kanamycin-resistance gene and a T7 phage RNA polymerase gene under the control of the *lac* promoter. Plasmid pT7BO4 carries an ampicillin-resistance gene (*bla*) and the T7 class III promoter followed by the bO gene, which encodes an N-terminal methionine codon and the amino acid sequence of bO (4, 6). To express bO, cells were grown in LB medium and induced by addition of 2 mM isopropyl β-D-thiogalactoside (Sigma) and then further grown for 1–2 hr. To obtain preparative amounts of bO, rifampicin was added to a concentration of 20 μg/ml 1–2 hr after induction, and the cells were grown for another 2 hr. Rifampicin did not influence localization and folding of bO, the fusion protein of bO with *E. coli* outer membrane protein (Omp-bO), the fusion protein of bO with *E. coli* lipoprotein (Lpp-bO), or bO with the *H. halobium* presequence (Hh-bO).

Methods. Experimental procedures for immunoprecipitation of bO from SDS-solubilized membranes (4) and purification of bO polypeptides from dry membranes were as described (7). For immunoprecipitation, polyclonal anti-bO antibody was used, whereas, for immunoblots, monoclonal antibody BR114 (8) was used.

Signal Peptide Cleavage *in Vitro*. Solubilized *E. coli* signal peptidase I (1 mg/ml) was a gift from William Wickner (University of California, Los Angeles). Omp-bO (20–50 μg) in 100 μl of 1% dimyristoyl phosphatidylcholine/1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/50 mM sodium phosphate buffer, pH 7.0, was incubated with 2–10 μg of the leader peptidase at 23°C for 4 hr.

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Abbreviations: bR, bacteriorhodopsin; bO, bacterio-opsin; Hh-bO, bacterio-opsin with the *Halobacterium halobium* presequence; Omp-bO, fusion protein of bO with *Escherichia coli* outer membrane protein; Lpp-bO, fusion protein of bO with *E. coli* lipoprotein.

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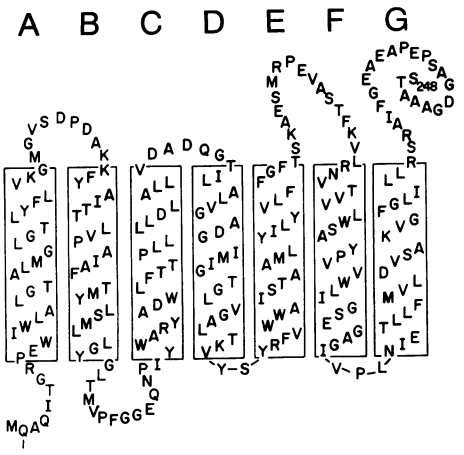

FIG. 1. A model for the secondary structure of bR as present in the purple membrane. Retinal is attached to Lys-216 located in transmembrane helix G.

Labeling of Lpp-bO with [^{3}H]Palmitic Acid. For palmitic acid labeling of Lpp-bO, exponentially growing *E. coli* W3110 cells were exposed to 25 μCi (1 Ci = 37 GBq) of [^{3}H]palmitic acid (30 Ci/mmol; NEN) per ml for 20 min. Globomycin (100 μg/ml) was added to the culture 10 min before induction.

Location of bO in Cells and in Membrane Fractions. *(i) Alkaline treatment of* E. coli *cells.* Induced cells were incubated with 0.1 M NaOH on ice for 30 min and then centrifuged for 15 min to separate the alkali-soluble and -insoluble fractions (9). Both fractions were precipitated by trichloracetic acid and analyzed by immunoblotting.

(ii) Total membrane preparation. The total membrane fraction was prepared by lysozyme/EDTA treatment followed by osmotic lysis and further ultracentrifugation at 210,000 × *g* for 2.5 hr as described (10). bO in the fractions was analyzed by immunoblotting.

(iii) Flotation sucrose density gradient centrifugation. Total membranes (670 μg of protein), prepared as above, were suspended in 2 ml of 58% (wt/wt) sucrose/5 mM EDTA/10 mM Tris acetate, pH 7.8, and the mixture was applied on a 0.5-ml cushion of 67% (wt/wt) sucrose at the bottom of a tube. After centrifugation at 36,000 rpm in a Beckman SW40 rotor for 70 hr, the membrane fractions at different densities were collected in 40 fractions from the bottom to the top (11).

(iv) Sucrose density gradient centrifugation. The total membranes prepared as above were further fractionated on a sucrose gradient (10), and fractions were assayed as described above.

(v) Triton/Mg^{2+} solubilization. The total membranes (100–500 μg of protein) were incubated in 100 ml of 2% (vol/vol) Triton X-100/30 mM $MgCl_2$/50 mM Tris·HCl, pH 8.0, at 23°C for 20 min. After centrifugation at 18,000 × *g* for 30 min at 4°C, the pellets were suspended in the above buffer, incubated again, and centrifuged (12, 13). The first and second soluble fractions and the insoluble fraction were analyzed.

[^{3}H]Retinal Binding. All-*trans*-[15-^{3}H]retinal (1.1 Ci/mmol) was prepared from all-*trans*-retinal (Sigma) and NaB^3H_4 (20.9 Ci/mmol; Amersham) as described (14). This was used in binding to bO and related proteins under regeneration conditions.

(i) [^{3}H]Retinal binding in vitro. [^{3}H]Retinal in ethanol was added to 0.2–0.7 μM cytoplasmic membranes suspended in buffer at pH 6.0, and the membranes were incubated at room temperature for 2 hr in the dark. After centrifugation, the membranes were suspended in 0.5 ml of 2 M Tris·HCl (pH 8.0). To the suspension was added 2.5 mg of $NaBH_4$ in 25 μl of H_2O every 15 min for 1 hr at 0°C under the light of a Kodak model 800 slide projector equipped with a Schott OG530 filter. After dilution with 4 ml of 50 mM phosphate buffer (pH 6.0), the membranes were collected by centrifugation, analyzed by SDS/PAGE, and visualized by fluorography. [^{3}H]Retinal binding without $NaBH_4$ treatment did not give any positive bands by fluorography.

(ii) [^{3}H]Retinal binding in vivo. [^{3}H]Retinal (1 μM) was added to a culture of *E. coli* cells 30 min before induction. Two hours after induction, cells were harvested and the total membranes were prepared and further treated with $NaBH_4$ as described above. [^{3}H]Retinal-labeled bO from purple membrane was obtained as described earlier (15).

Purification and CNBr Cleavage of [^{3}H]Retinyl-bO from *E. coli* Membrane. *E. coli* membranes containing [^{3}H]retinyl-bO reduced with $NaBH_4$ as described above were then purified by BR114 immunoaffinity column chromatography or preparative SDS/PAGE. The CNBr cleavage of purified [^{3}H]-retinyl-bO was carried out, and cleavage products were analyzed by SDS/7 M urea/12.5% PAGE (14).

Regeneration of bR with *E. coli* Phospholipids. *E. coli* crude phospholipids were prepared from strain W3110 according to Bligh and Dyer (16) and used instead of dimyristoyl phosphatidylcholine in standard regeneration of bO (15).

RESULTS

Expression of bO, Omp-bO, Lpp-bO, and Hh-bO in *E. coli*. bO, Omp-bO, Lpp-bO, and Hh-bO (Fig. 2) were expressed by the procedure described previously (4, 17). As determined earlier, >70% of bO, but only about 20% of the expressed Omp-bO and Lpp-bO, was degraded in *E. coli*. Hh-bO was degraded extensively. The proteins formed are shown in Fig. 3.

Characterization of the *in Vivo* Expression Products: Limited Cleavage of Signal Sequences. The signal sequence of Omp-bOI was not cleaved in *E. coli*, whereas the signal sequence of Omp-bOII was cleaved only to an extent of 5–10% (Fig. 3*A*). The low level of processing in the Omp-bOII fusion protein could be due to the lack of proper translocation of the signal sequence across the cytoplasmic membrane or due to the lack of the structure required for recognition by the signal peptidase. Processing of the signal sequences by the purified signal peptidase I was studied in detergent micelles (18, 19). No cleavage of the signal sequence in Omp-bOI was observed. On the other hand, Omp-bOII was cleaved com-

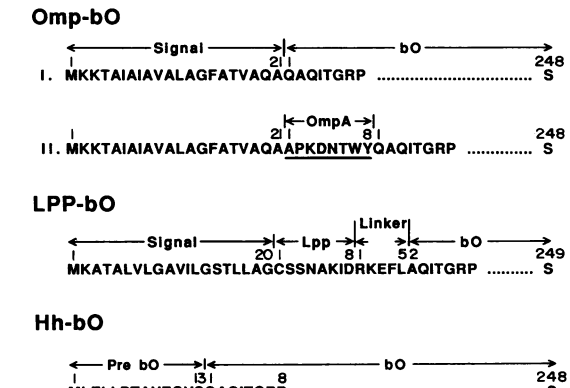

FIG. 2. Sequence of the leader peptide and the linker segments attached at the N terminus of bO. Mature bO (Gln-1 to Ser-248) contains only an N-terminal formylmethionine when expressed in *E. coli*. Omp-bOI contains a fusion of the OmpA signal peptide sequence with mature bO, and Omp-bOII contains the signal and 8 amino acids of mature OmpA fused to bO. In Lpp-bO, mature bO (Ala-2 to Asp-249) is present. The Cys-20 residue gets modified with a dipalmitoylglyceryl moiety. Hh-bO contains the 13-amino acid presequence that is present in the *bop* gene of *H. halobium*. The anticipated cleavage site for signal peptidase is shown by the horizontal arrow in each construct.

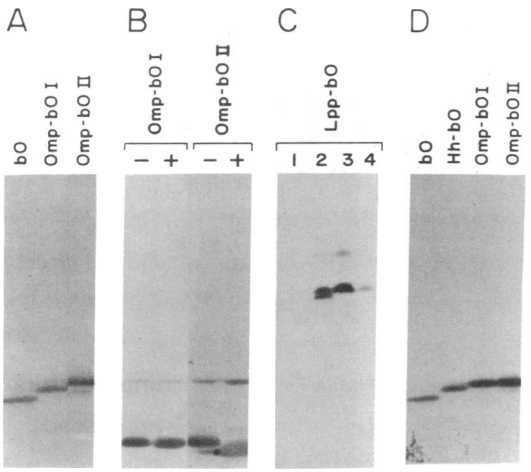

FIG. 3. Expression and processing of bO, Omp-bO, Lpp-bO, and Hh-bO. (*A*) bO and its fusion proteins, Omp-bOI and Omp-bOII, expressed in *E. coli* were visualized by immunoblotting. (*B*) Cleavage *in vitro* of Omp-bO polypeptide by signal peptidase I. In each case the protein incubated with (+) and without (−) leader peptidase is shown. (*C*) The immunoprecipitate of [^{3}H]palmitate-labeled Lpp-bO with rabbit polyclonal anti-bR antibody is shown. For labeling *E. coli* with the vector, pIN-bO3 in the presence of 2 mM isopropyl β-D-thiogalactoside and in the presence of isopropyl β-D-thiogalactoside and globomycin (100 μg/ml) were used. The immunoprecipitates with nonimmune serum (lane 1) and with anti-bR-antibody (lane 2) of Lpp-bO are shown. Inhibition of processing by globomycin for 10 min (lane 3) and 30 min (lane 4) was followed. (*D*) Immunoblot of Hh-bO expressed in *E. coli* is compared with bO, Omp-bOI, and Omp-bOII.

pletely (Fig. 3*B*). Further, in a mixture of Omp-bOI and Omp-bOII, only Omp-bOII was cleaved by the signal peptidase. It therefore appears that the failure to cleave Omp-bOI is due to the incompatible structure generated by the fusion of Omp with bO. Further, the partial cleavage *in vivo* of Omp-bOII must be due to incomplete translocation across the cytoplasmic membrane.

Cleavage of Lpp Signal Sequence Is Partial. The cleavage of the Lpp signal sequence in *E. coli* occurs in several steps, which involve modification and acylation at Cys-20 (20). The process can be followed with radioactive fatty acids. When *E. coli* cells expressing Lpp-bO (17) were grown in a minimal medium supplemented with [^{3}H]palmitic acid, Lpp-bO appeared as a doublet (Fig. 3*C*). The two bands showed a precursor–product relation in pulse–chase experiments with palmitic acid. The formation of the processed band was completely abolished by globomycin, which inhibits the signal peptidase II (20, 21). The peptidase II is specific for processing the signal sequence of *E. coli* lipoprotein (Lpp).

We conclude that Cys-20 in the Lpp signal sequence is modified normally but that the cleavage of the signal sequence is only about 50% (Fig. 3*C*) and processed bO retains the eight N-terminal Lpp amino acids as well as the five linker amino acids.

The Presequence in Hh-bO Is Not Processed in *E. coli*. The signal sequences investigated above in bO expression were heterologous to the archaebacterial *bop* gene. As a contrast, expression of the *bop* gene with the native presequence in *bop* gene was carried out. Hh-bO, containing the 13-amino acid presequence (Fig. 2), was extensively degraded (about 80% of total) in *E. coli*. The presequence was not processed in the protein that survived (Fig. 3*D*).

The Expressed bO Proteins Are Located in *E. coli* Membranes. Three different techniques were applied to investigate the location of bO and modified bO polypeptides. First, ultracentrifugation of *E. coli* cell lysates yielded membrane pellets containing all of the bO proteins expressed. Further, when *E. coli* expressing bO was treated with alkali and the mixture was centrifuged, bO was recovered mostly in the pellet fraction. Further evidence that bO was in the membrane was obtained by flotation sucrose gradient centrifugation (22) in which bO floated with the membranes from high density to low density.

bO in *E. coli* Is in Both Cytoplasmic and Outer Membranes. Localization of bO in *E. coli* membranes was further studied using the following two methods for the separation of the cytoplasmic and outer membranes. The total *E. coli* membranes were separated by sucrose density gradient centrifugation analysis (10). The results are shown in Fig. 4*A*. bO was present in both the outer membrane (fractions 6–10) and the cytoplasmic membrane (fractions 23–30) fractions.

Although the sucrose gradient centrifugation experiment of Fig. 4*A* gave clear separation of cytoplasmic and outer membrane, the possibility that an overproduced, hydrophobic, foreign protein such as bO in *E. coli* might behave differently from endogeneous membrane proteins existed. The experiment with the *E. coli* membrane containing Lpp-bO excludes this possibility. Membranes from Lpp-bO

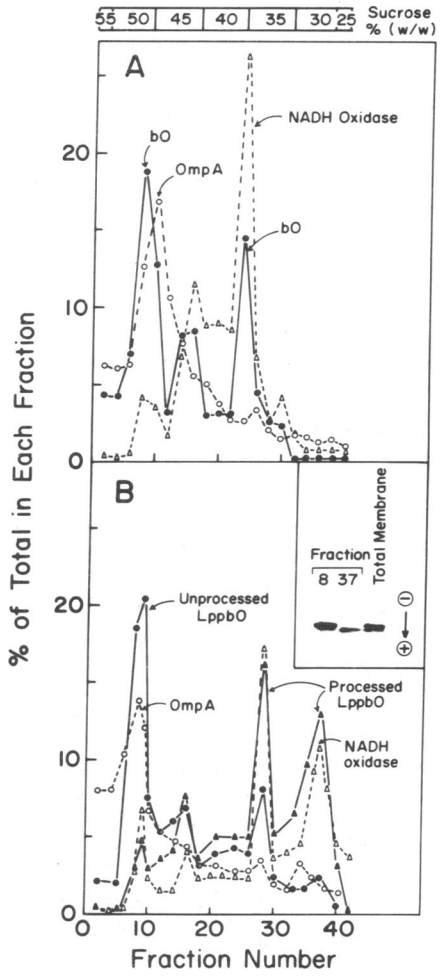

FIG. 4. Isopycnic sucrose density gradient centrifugation of the *E. coli* total membranes containing bO (*A*) and Lpp-bO (*B*). The sucrose concentration of the stepwise gradient is given at the top. The total membrane samples were layered on the top of the gradient and subjected to centrifugation and analyzed as described in *Materials and Methods*. NADH oxidase is a marker for the *E. coli* inner membrane and OmpA is a marker for the outer membrane. In *B*, the distributions of processed Lpp-bO (△) and unprocessed Lpp-bO (○) are shown separately, and the *Inset* shows the results of immunoblotting following SDS/PAGE of fractions 8 (outer membrane) and 37 (cytoplasmic membrane) and total membrane.

expressed in *E. coli* contain a mixture of processed and unprocessed Lpp-bO. As shown in Fig. 4*B*, unprocessed Lpp-bO was distributed between the outer and the cytoplasmic membrane. Outer membrane contained 22% of the unprocessed Lpp-bO and no processed Lpp-bO. The cytoplasmic membrane fraction also contained unprocessed form but all of processed form of Lpp-bO. The processed form corresponded to 70% of the Lpp-bO in the cytoplasmic membrane. This indicates that the isopycnic sucrose density gradient gives the outer membrane essentially free of the cytoplasmic membrane. Thus, the bO without a signal sequence observed in the outer membrane fraction is not due to the contamination of the outer membrane with the cytoplasmic membrane, which has been found in the case of some other foreign proteins expressed in *E. coli* (23). Further, Omp-bOII, which was also processed partially, but contained about 10-fold more unprocessed protein, behaved like Lpp-bO.

It is known that, of the two membranes of *E. coli*, the cytoplasmic membrane can be selectively solubilized by Triton X-100 in the presence of Mg^{2+} (12, 13). The total *E. coli* membranes were separated into Triton X-100-soluble and -insoluble fractions, and localization of bO was tested. Examination by SDS/PAGE showed a number of proteins with a wide range of molecular weights in the soluble fraction. The insoluble fraction contained the typical outer membrane proteins, such as OmpA and OmpF/C. The distribution of bO in the two membrane fractions was analyzed. Triton X-100 solubilized 30–45% of the total bO while the rest was in the insoluble pellet, indicating that bO was present in both of the two membranes. Fractionation of Lpp-bO expressing cell membranes showed that the cytoplasmic membrane contained essentially all of processed Lpp-bO and some unprocessed Lpp-bO. The Triton-insoluble fraction contained only unprocessed Lpp-bO (data not shown).

Binding of Retinal to Lys-216 of bO in *E. coli* Membranes. Does the bO in *E. coli* membrane fold so as to bind all-*trans*-retinal and to form a Schiff base with Lys-216? Retinal binding to bO in growing cells and in fractionated cytoplasmic membrane was examined by reduction of the Schiff base linkage with $NaBH_4$ by the procedures described earlier (14).

The cytoplasmic membrane preparation contained bO at a level of 1% of the total membrane protein. Although a number of radioactively labeled bands formed the background, the presence of a strong radioactive band at a position corresponding to bO was unambiguous (Fig. 5). This band was characterized as bO as follows. (*i*) When the same experiment was carried out with Omp-bOI or Lpp-bO, the mobility of this radioactive band corresponded to that of Omp-bOI and Lpp-bO, which was slower than bO. (*ii*) The protein in the labeled bO band could be purified by immunoaffinity chromatography (17) with the monoclonal anti-bO antibody (BR114) (9). (*iii*) Gel-purified protein from this band showed a CNBr cleavage pattern identical to that given by bO (14). Similar characterizations were done for all modified bO fusion proteins as well.

The efficiency of [^{3}H]retinal binding to bO was about 1%, based on the ^{3}H-specific activity and the amount of bO determined by immunoblotting. The bO band bound about 2% retinal in the cytoplasmic membrane fraction while the outer membrane fraction bound about 0.7% retinal (Fig. 5). Addition of retinal to whole cells yielded few other labeled proteins in addition to bO.

E. coli cells expressing Lpp-bO were grown in the presence of exogeneously supplied all-*trans*-[^{3}H]retinal and treated with $NaBH_4$ to reduce the retinylidene Schiff base. Fractionation of the membranes showed that the protein carrying [^{3}H]retinal was located predominantly in the cytoplasmic membrane and its mobility on SDS gels corresponded to that of the processed Lpp-bO (Fig. 5). The amount of processed

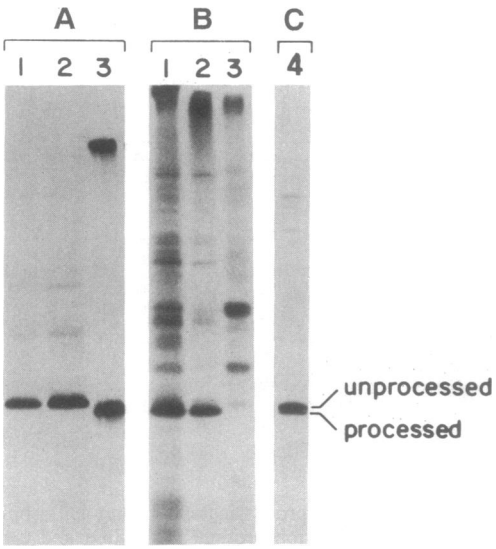

FIG. 5. Binding of [^{3}H]-all-*trans*-retinal to Lpp-bO *in situ*. (*A* and *B*) Binding of [^{3}H]retinal to bO in two preparations of *E. coli* cytoplasmic membrane *in vitro*. Lane 1, [^{3}H]retinyl-bO prepared from purple membrane; lanes 2 and 3, 20 μg and 40 μg of *E. coli* membrane protein after [^{3}H]retinal binding and $NaBH_4$ reduction. (*C*) Lane 4, immunoblot of Lpp-bO.

Lpp-bO was 30% of the total Lpp-bO and of this only about 20% bound retinal. Less than 1% of the unprocessed Lpp-bO bound retinal. Similar experiments and estimation of retinal binding indicated that 40% of processed Omp-bOII and 0.8% of unprocessed Omp-bOII in the cytoplasmic membrane fraction bound the chromophore. The fraction of the unprocessed Omp-bOII in the outer membrane that bound retinal was about 1% (data not shown).

Attachment Site of [^{3}H]Retinal to bO in *E. coli* Is Lys-216. Previously, the binding site of retinal in native bO has been shown to be Lys-216 by CNBr degradation of the $NaBH_4$-reduced bR followed by characterization of the C-terminal polypeptide (amino acid 210 through amino acid 248) (14). In the present work, [^{3}H]retinyl-bO in *E. coli* membranes was purified by immunoaffinity chromatography and reduced with $NaBH_4$. Analysis of CNBr cleavage peptides by SDS/7 M urea/12.5% PAGE and subsequent fluorography showed that, like the native bO, bO from the *E. coli* membranes contained the radioactivity originating from [^{3}H]retinal in the C-terminal cyanogen bromide-generated peptide CN6 (residues 210–248). Since Lys-216 is the only lysine in this fragment, the binding site of [^{3}H]retinal in bO must be Lys-216 (data not shown).

DISCUSSION

A light-driven proton pump inserted into the *E. coli* cytoplasmic membrane in the correct orientation would open up a variety of opportunities for biophysical studies, including a simple method for screening of bR mutants. With these aims, we studied systematically (*i*) the expression of the *bop* gene with and without *E. coli* signal sequences as well as the expression of the *bop* gene containing the *H. halobium* presequence, (*ii*) the location of the expressed products in the cell, and (*iii*) the binding of all-*trans*-retinal to obtain functional bacteriorhodopsin, which would show that in *E. coli* the opsin folds as it does in *H. halobium* to form the retinal binding pocket.

The *bop* gene was first expressed without an N-terminal signal sequence except for the initiator methionine codon (4) (Fig. 1). This situation is analogous to a number of *E. coli* integral membrane proteins that are expressed without signal

sequences and are integrated into the cytoplasmic membrane. Degradation of expressed bO was extensive (4), but the fraction that survived was homogeneous; surprisingly, it was distributed between the cytoplasmic and the outer membrane, and only a very small fraction (1–2%) of the total protein bound retinal.

Expression of the *bop* gene was next studied after adding *E. coli* OmpA and the Lpp signal sequences (Fig. 2). Both signal sequences protected the expression products from degradation and, in both cases, the products were distributed between the inner and outer membranes. The OmpA sequence in Omp-bOI was not cleaved at all. The use of the modified OmpA signal sequence (Omp-bOII) led only to partial (15%) cleavage of the signal sequence even though, *in vitro*, the signal peptidase cleaved the Omp-bOII sequence completely to form bO. The processed bO formed *in vivo* from Omp-bOII was exclusively in the cytoplasmic membrane as found for the processed Lpp-bO. The unprocessed Omp-bO bound retinal again to only 1–2%, whereas the processed bO bound retinal to about 20%. This showed that the processed bO was present in at least two differently folded states.

The Lpp signal sequence is specifically cleaved by signal peptidase II (21). Ghrayeb and Inouye (24) showed that for the cleavage of the Lpp signal sequence only the eight amino acids at the N terminus of the Lpp structural gene were required (24) for the action of the peptidase. On expression of the Lpp-bO construct (Fig. 2), about 50% of the Lpp signal sequence was cleaved; the processed bO contained 13 extra amino acids at the N terminus (Fig. 2). All of the processed bO was in the cytoplasmic membrane, but only 20% of it bound retinal. Thus, as found above for the Omp-bOII protein, the protein existed in two differently folded states.

The possibility was tested that the *H. halobium* bR presequence (Fig. 2) might have a decisive influence on the folding of the transmembrane protein. The *bop* gene with the presequence was expressed in *E. coli*. As described above, degradation of the expressed protein was extensive, and its behavior, location, and low retinal binding were very similar to those described above for other bO proteins. Finally, the possibility was considered that the very low binding of retinal to bO and related proteins expressed in *E. coli* might be due to the phospholipids in these membranes. *E. coli* lipids were substituted in the standard *in vitro* procedure for folding and regeneration of bR (see *Materials and Methods*). *E. coli* phospholipids were fully effective in bR folding, retinal binding, and regenerating the bR-like chromophore.

Thus, a major surprise in the expression of bO in *E. coli* has been that bO does not fold to the conformation that is present in *H. halobium* and that binds retinal. In *in vitro* procedures using detergent/phospholipid micelles, bO and a large variety of its mutants have been shown to fold to the conformation that binds retinal. There is clearly a difference between the *in vitro* reconstitution in which one starts with a completed molecule and presents it to a detergent/phospholipid mileu and the *in vivo* situation in which the protein is inserted into the membrane by mechanisms not well understood (25, 26). Genetic studies in *E. coli* on the secretion and transport of *E. coli* periplasmic and outer membrane proteins have demonstrated the involvement of a number of secretory gene products (27–29). Although requirements of such factors for the insertion and folding of polytopic transmembrane proteins such as bO are not known, it seems likely that one reason for the present results could be the lack of recognition of our proteins by one or more of the secretory factors.

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1. Stoeckenius, W., Lozier, R. H. & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta* **505**, 215–278.
2. Khorana, H. G. (1988) *J. Biol. Chem.* **263**, 7439–7442.
3. Chang, S. H., Majumdar, A., Dunn, R., Makabe, O., RajBhandary, U. L., Khorana, H. G., Otsuka, E., Tanaka, T., Taniyama, Y. & Ikehara, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3398–3402.
4. Karnik, S. S., Nassal, M., Doi, T., Jay, E., Sgaramella, V. & Khorana, H. G. (1987) *J. Biol. Chem.* **262**, 9255–9263.
5. Tabor, S. & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1074–1078.
6. Khorana, H. G., Braiman, M. S., Chao, B. H., Doi, T., Flitsch, S., Gilles-Gonzalez, M. A., Hackett, N. R., Karnik, S. S., Mogi, T., Nassal, M. M. & Stern, L. J. (1987) *Chemica Scripta* **27B**, 137–147.
7. Braiman, M., Stern, L. J., Chao, B. H. & Khorana, H. G. (1987) *J. Biol. Chem.* **262**, 9271–9276.
8. Kimura, K., Mason, T. L. & Khorana, H. G. (1982) *J. Biol. Chem.* **257**, 2859–2867.
9. Russel, M. & Model, P. (1982) *Cell* **28**, 177–184.
10. Osborn, M. J., Gander, J. E., Parisi, E. & Carson, J. (1972) *J. Biol. Chem.* **247**, 3962–3972.
11. Ishidate, K., Creeger, E. S., Zrike, J., Deb, S., Glauner, B., McAlister, T. J. & Rothfield, L. I. (1986) *J. Biol. Chem.* **261**, 428–443.
12. DePamphilis, M. L. & Adler, J. (1971) *J. Bacteriol.* **105**, 396–407.
13. Schnaitman, C. A. (1971) *J. Bacteriol.* **108**, 545–552.
14. Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A. H., Takagaki, Y. & Khorana, H. G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2225–2229.
15. Liao, M.-J., London, E. & Khorana, H. G. (1983) *J. Biol. Chem.* **258**, 9949–9955.
16. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **39**, 911–917.
17. Dunn, R. J., Hackett, N. R., McCoy, J. M., Chao, B. H., Kimura, K. & Khorana, H. G. (1987) *J. Biol. Chem.* **262**, 9246–9254.
18. Zimmerman, R., Watts, C. & Wickner, W. (1982) *J. Biol. Chem.* **257**, 6529–6536.
19. Wolfe, P. B., Silver, P. & Wickner, W. (1982) *J. Biol. Chem.* **257**, 7898–7902.
20. Hantke, K. & Braun, V. (1973) *Eur. J. Biochem.* **34**, 284–296.
21. Inukai, M., Takeuchi, M., Shimizu, K. & Arai, M. (1978) *J. Antibiot.* **31**, 1203–1205.
22. Hirst, T. J., Randall, L. L. & Hardy, S. J. S. (1984) *J. Bacteriol.* **157**, 637–642.
23. Tomassen, J., Leunissen, J., van Damme-Jongsten, M. & Overduin, P. (1985) *EMBO J.* **4**, 1041–1047.
24. Ghrayeb, J. & Inouye, M. (1984) *J. Biol. Chem.* **259**, 463–467.
25. Lee, C. & Beckwith, J. (1986) *Annu. Rev. Cell. Biol.* **2**, 315–336.
26. Randall, L. L., Hardy, S. J. S. & Thom, J. R. (1987) *Annu. Rev. Microbiol.* **41**, 507–541.
27. Cabelli, R. J., Chen, L., Tai, P. C. & Oliver, D. B. (1988) *Cell* **55**, 683–692.
28. Collier, D. N., Bankaitis, V. A., Weiss, J. B. & Bassford, P. J., Jr. (1988) *Cell* **53**, 273–283.
29. Crooke, E., Guthrie, B., Lecker, S., Lill, R. & Wickner, W. (1988) *Cell* **54**, 1003–1011.
30. Stern, L. J. & Khorana, H. G. (1989) *J. Biol. Chem.* **264**, 14202–14208.