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Wild-Type and Mutant Bacterioopsins D85N, D96N, and R82Q: High-Level Expression in *Escherichia coli*†

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ABSTRACT: The integral membrane protein bacterioopsin, found in the extremely halophilic archaebacterium *Halobacterium halobium*, was expressed in *Escherichia coli* as a fusion protein containing 13 heterologous amino acids at the amino terminus. The expressed protein was localized primarily to the *E. coli* cytoplasmic membrane (>80%) and had an in vivo half-life of 26 min. The amount of bacterioopsin in *E. coli* crude lysates was quantitated immunologically from Western blots and was expressed at 10–20-fold higher levels than seen previously (i.e., 17 mg/L; 5.6% of the total protein). Three distinct forms of the protein were detected immunologically: two of the forms were generated by the removal of either one or four amino acid residues at the amino terminus; the third form remained unaltered.

Bacterioopsin (BO)[1] complexed with the chromophore retinal constitutes bacteriorhodopsin (BR) in the purple membrane (PM) of *Halobacterium halobium* (Stoeckenius & Bogomolni, 1982). Under conditions of low oxygen tension and high light intensity, BR pumps protons across the cell membrane as it cycles through a number of short-lived (femtosecond to millisecond) photointermediates (Oesterhelt & Stoeckenius, 1973). The resultant electrochemical gradient is used to drive energy-requiring metabolic processes (e.g., ATP synthesis) and provides sufficient energy to sustain phototrophic growth (Oesterhelt & Krippahl, 1983). The formation of highly ordered two-dimensional crystalline patches of BR in the purple membrane has facilitated structural analyses which have been performed by electron imaging to about 3.5 Å in-plane by ~10 Å perpendicular to the membrane plane (Henderson et al., 1990).

In vivo experimental approaches involving alteration of specific amino acids of BO to determine which residues are responsible for proton pumping have been hampered by the high spontaneous mutation frequencies found in *H. halobium* (Pfeifer et al., 1981). Spontaneous purple membrane deficient mutants[2] occur at a frequency of 10^{-4} and with few exceptions are due to the integration of insertion elements in or near the bacterioopsin (*bop*) gene (Betlach et al., 1984). However, mutagenic agents have been used successfully in vivo to produce point mutations in the purple membrane producing halobacterial strain GRB (Soppa & Oesterhelt, 1989; Soppa et al., 1989). Strain GRB has a much lower frequency of spontaneous *bop* mutations than *H. halobium* and lacks all

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[1] Abbreviations: BO, bacterioopsin; BR, bacteriorhodopsin; PM, purple membrane; e-BO, bacterioopsin expressed from pβgbop in *E. coli*; e-BR, e-BO complexed with retinal; IPTG, isopropyl β-D-thiogalactopyranoside; SMG, supplemented M9 glycerol; bp, base pair(s); kbp, kilobase pair(s); PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Mutant: A "mutant" is a strain carrying one or more mutations. For clarity and consistency with overwhelming usage within the literature, "mutant bacterioopsin", as used in the title, is defined as a bacterioopsin protein containing a substituted amino acid residue.

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insertion elements characterized to date (Ebert et al., 1986). These GRB *bop* mutants were isolated by using a counterselection which exploited their inability to sustain phototrophic growth. Bop mutants with substitutions at six locations were found. Of these, alterations at Asp-85 and Asp-96 produced the most profound effects on function.

Mutations in the *bop* gene generated in vitro cannot be introduced and expressed in *H. halobium* until a practical and efficient DNA transformation system becomes available for the extremely halophilic archaebacteria. Although transformation of an *H. halobium* strain carrying an insertion in the *bop* gene with a plasmid carrying the wild-type *bop* gene has been demonstrated (Ni et al., 1990), the amount of BR expressed from these transformed cells (~1.3 mg of BR/L) is about 10-fold lower than in this report, and 20-fold lower than in the *H. halobium* strains from which purple membrane is commonly purified (Miercke et al., 1989a). Thus, overexpressing site-directed *bop* mutations in heterologous systems continues to be an effective approach until transformation and *bop* expression levels in the homologous *H. halobium* system become more tractable. Bacterioopsin has been expressed in yeast (Hildebrandt et al., 1989), but analyses of purified bacterioopsins containing altered amino acid residues have yet to be reported in this system. Both wild-type BO (Karnik et al., 1987) and bacterioopsins containing in vitro generated point mutations have been expressed in *Escherichia coli* [reviewed in Khorana et al. (1987)], but at low levels (Nassal et al., 1987). Perturbations in the pumping and photocycle of BR molecules containing single amino acid substitution mutations expressed in *E. coli* have been reported, and several functionally important residues have been elucidated. Asp-96 has been implicated in the reprotonation of the Schiff base (Otto et al., 1989) whereas Asp-85, Asp-212, and Arg-82 appear to be involved in the Schiff base deprotonation and proton release early in the photocycle (Otto et al., 1990).

To further refine the interactions of these and other residues, the structure of BR proteins containing substituted amino acids must also be analyzed. Such studies require large amounts of purified protein. This work describes an efficient *E. coli* expression system for the *bop* gene that produces the highest levels of expressed BO yet reported. As described in the following paper (Miercke et al., 1991), this expressed BO is functionally indistinguishable from BO isolated from *H. halobium* and after retinylation can be crystallized into two-dimensional arrays.

Materials and Methods

Reagents. Restriction endonucleases and T4 DNA ligase were from New England Biolabs, Beverly, MA. S1 nuclease was obtained from Boehringer Mannheim, Indianapolis, IN. Klenow fragment of DNA polymerase I was from Bethesda Research Labs, Gaithersburg, MD. [γ-^{32}P]ATP (2000–3000 Ci/mM), deoxynucleotide [α-^{32}P]triphosphates (400 Ci/mM), and L-[^{35}S]methionine (1337 Ci/mmol, in vivo cell labeling grade) were obtained from Amersham, Arlington Heights, IL. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were from P-L Biochemicals, Milwaukee, WI.

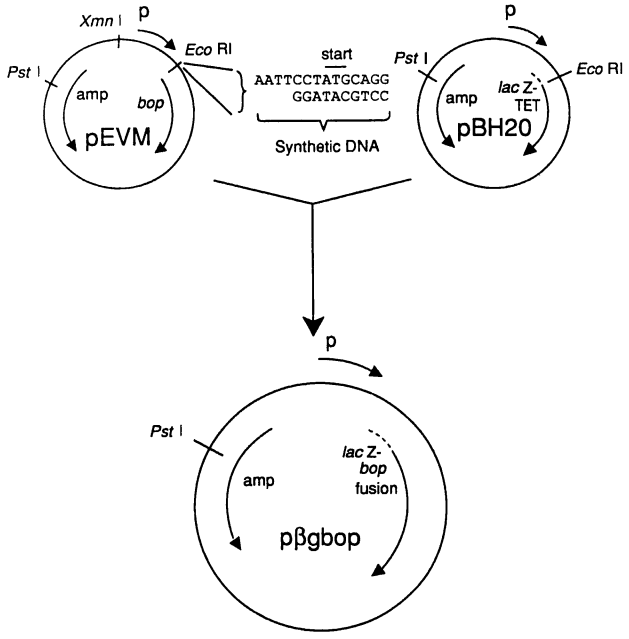
Bacterial Strains, Media, and Growth Conditions. Escherichia coli K12 strains were the HB101 derivative D12·10 (*leuB6*, *proA2*, *recA13*, *lacY1*, *ara14*, *galK2*, *xyl5*, *mtl1*, *rpsL20*, *supE44*, *hsdS20*, *lacI*^{q}, λ^-, F^-, Kuhn et al., 1986); NCM533 (*lacZ*::Tn5, *lacI*^{q1}, λ^+; provided by J. Keener and S. Kustu, University of California, Berkeley); JM105 [*thi*, *rpsL*, *endA*, *sbcB15*, *hspR4*, Δ(*lac-proAB*), (F′, *traD36*, *proAB*, *lacI*^{q}ZΔ*M15*); grown as specified in Yanisch-Perron et al., (1985)]; and CJ236 [*dut1*, *ung1*, *thi1*, *relA1*/pCJ105-(cm^r); Kunkel et al., 1987] (provided by T. Kunkel, National Institute of Environmental Health Sciences, Research Triangle Park, NC). Complex medium was YT (Miller, 1972), and minimal medium was M9 salts (Davis et al., 1980) containing 0.4% (v/v) glycerol and supplemented with 1.0 μM $FeCl_3$ and 0.2 mg/mL thiamin (SMG medium). Growth was monitored spectrophotometrically using a Beckman DU-50 spectrophotometer, and, unless otherwise indicated, BO expression was induced at an OD_{600} of 0.8 by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM. Cells were harvested by centrifugation (9500*g* for 25 min, 4 °C) 45-min postinduction, and cell pellets were resuspended in ~10 mL of lysis buffer (50 mM sodium phosphate, pH 7.2, and 0.025% NaN_3) per liter of cells harvested. DNase I and RNase A (Sigma Chemical Co., St. Louis, MO) were added at 2 μg/mL and the cells lysed by two passes through a French press at 6000 psi.

Expression Vector Constructions. The following constructions are depicted in Figure 1. A 952 base pair (bp) *Nae*I fragment containing all but 24 bp of the 3′ terminus of the *bop* gene was cloned into a filled-in *Xma*I site in the *lamB* gene of pHSF1 (a pBR322 derivative with the *lamB* gene cloned into the *Eco*RI/*Bam*HI sites, obtained from J. Hedgpeth, Codon, Inc., Brisbane, CA). A 4.6 kbp *Eco*RI/*Hae*III fragment containing the *bop* gene (lacking four 5′-terminal bp) was gel-purified and ligated to a synthetic fragment of DNA which contained the missing four residues, an ATG start codon and half of an *Eco*RI site (Figure 1A). Nucleotide sequence analysis of an ampicillin-resistant transformant confirmed the existence of an *Eco*RI site, 6 bp upstream of the *bop* gene, and an ATG start codon adjacent to the CAG codon encoding the first residue of the mature form of BO found in *H. halobium*. The 3′ terminus of the *bop* gene was reconstructed by replacement of the *Kpn*I/*Sma*I fragment in the vector with a *Kpn*I/filled-in *Bst*EII fragment from the wild-type *bop* gene. The resulting intact *bop* gene was placed under control of a tandem *lacUV5* promoter by inserting a 300 bp *Eco*RI fragment containing the tandem promoter (isolated from pKB268; Backman & Ptashne, 1978) into the *Eco*RI site upstream of the *bop* gene in the expression vector (designated pEVM, Figure 1A).

The pEVM plasmid vector was also used in the construction of a β-galactosidase–bacterioopsin protein fusion vector (pβgbop; see Figure 1A). This vector was designed to encode 13 heterologous residues (Figure 1B) fused to the amino terminus of BO with expression of the fusion under the control of the wild-type *lac* promoter. A *Pst*I/filled-in *Eco*RI fragment (~4000 bp) containing the *bop* gene from pEVM and a *Pst*I/filled-in *Eco*RI fragment (~1000 bp) containing the wild-type *lac* promoter, the ribosomal binding site, and 25 bp of the 5′-terminal nucleotide sequence of the *lacZ* gene from pBH20 (Heyneker et al. 1977) were gel-purified, ligated, and transformed into *E. coli* strain D12·10. One transformant lacking an *Eco*RI site was chosen and subjected to nucleotide sequence analysis, yielding the expected sequence, part of which is shown in Figure 1B.

Mutagenesis. Site-directed mutagenesis was performed with M13 phage cloning vectors (Yanisch-Perron et al., 1985) and the procedure of Kunkel et al. (1987). A 1400 *Bst*EII (filled-in)/*Bam*HI fragment containing the *bop* gene was cloned into the *Bam*HI/*Sma*I site of M13mp19 (Yanisch-Perron et al., 1985) and used as template for subsequent mutagenesis experiments. Synthetic oligonucleotides used to create the mutations were obtained from the Biomolecular Resource Center (University of California, San Francisco) and

A



B

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met leu glu leu leu pro thr ala val glu gly val ser gln ...

fmet thr met ile thr asp ser leu glu leu ile pro met gln ...
 ATG ACC ATG ATT ACG GAT TCA CTG GAA TTA ATT CCT ATG CAG ...
```

FIGURE 1: Bacterioopsin expression vectors. A description of these vectors and their construction appears under Materials and Methods and in Betlach et al. (1987). In *H. halobium*, processing of a BO precursor results in partial and/or complete removal of 13 amino-terminal residues and 1 carboxyl-terminal aspartic acid (Dunn et al. 1981). In all of the *E. coli* vector constructions, the *bop* gene has been engineered to encode a product lacking the 13 amino-terminal residues found in the precursor but including the carboxyl-terminal residue. (A) Schematic of vector constructions. Plasmids pEVM, pBH20, and pβgbop are indicated by circles. Relevant restriction sites used in the constructions are indicated. Arrows inside the circles denote genes encoding ampicillin resistance, BO, a β-galactosidase–tetracycline resistance fusion, and the β-galactosidase–bacterioopsin fusion. In the case of gene fusions, the dashed portion of the arrows represents 25 bp of the *lacZ* gene. The positions of the promoters used to regulate expression of the *bop* gene are indicated by a "P" above an arrow outside the circles. The *lacUV5* promoter is used in the pEVM vector whereas the *lac* promoter is used in the pBH2O and pβgbop vectors. The synthetic DNA fragment inserted at the 5′ terminus of the *bop* gene in pEVM is indicated. (B) The 13 amino acid precursor sequence (Dunn et al., 1987) and the first amino-terminal residue (Gln, in bold typeface) of the fully processed form of BO from *H. halobium* are given on the top line. The 13 heterologous amino acids encoded by the 5′ terminus of the pβgbop vector are shown on the bottom line with the corresponding nucleotide sequence located immediately below. The DNA encoding these 13 residues in the vector is followed by DNA encoding the fully processed form of BO found in *H. halobium*. Thus, the fourteenth residue encoded is a Gln residue (indicated in bold typeface).

consisted of a substitution of glutamine for arginine at position 82 (R82Q) and individual substitutions of asparagine for aspartic acid at positions 85 (D85N) and 96 (D96N). Following in vitro mutagenesis (Kunkel et al., 1987), the DNA reaction mixture was used to transfect competent JM105 cells. Mutants were identified by diagnostic restriction enzyme digestions and nucleotide sequence analysis (Sanger et al., 1977, Chen & Seeburg, 1985) of the double-stranded phage replicative form (RF) DNA.

Upon verification of the mutation, the entire miniscreen preparation was digested with *Kpn*I and *Ava*I, and a 180 bp fragment containing the mutation was gel-purified. This fragment was used to replace the corresponding wild-type fragment in the *E. coli* expression vector for BO, pβgbop. The presence of each mutation was confirmed by nucleotide sequence analysis. In addition, large cultures (e.g., 12 L) containing mutated bacterioopsins were checked for the presence of the appropriate mutation by DNA sequencing prior to purification.

Antibody Production and Purification. Rabbit polyclonal antibodies were raised against solubilized PM [2 mg/mL BR in 2% (w/v) octyl β-glucoside] mixed with Freund's complete adjuvent (equal volumes). Antisera were absorbed to crude lysates of *E. coli* NCM533. Lysates were prepared from cultures grown in SMG medium at 37 °C to an OD_{600} of ~1.0, harvested, resuspended in 5 mL of PBS (20 mM sodium phosphate and 0.15 M NaCl, pH 7.4) per gram of cells (wet weight), and sonicated. The lysate and immune serum were mixed in equal volumes and incubated overnight at 4 °C with constant mixing. Debris was removed by centrifugation at 128000*g* for 30 min at 4 °C and the absorbed serum stored at −60 °C.

Assay for Expression. Bacterioopsin expression was assayed immunologically on Western blots (Towbin et al., 1979) using polyclonal antibodies raised against solubilized PM (described above) as the primary antibody and alkaline phosphatase conjugated goat anti-rabbit as the secondary antibody. To reduce background and increase the sensitivity of the assay, we used poly(vinylidene difluoride) (PVDF) immobilon membranes (Millipore Corp., Bedford, MA) and modified an existing immunoassay procedure (Tsang et al., 1983) by blocking with 5% normal goat serum between the primary antibody step and exposure to the secondary antibody. Western blots were scanned at 530 nm using a Joyce-Loebl Ephortec densitometer in the transmission mode.

Isolation, Washing, and Fractionation of Crude Membranes. Cell pellets were converted to spheroplasts in the absence of $MgCl_2$ and centrifuged (Koshland & Botstein, 1980). The spheroplast pellet was resuspended in a minimal volume of ice-cold H_2O and subjected to two cycles of freezing and thawing. DNAse was added to a final concentration of 0.1 μg/mL and the lysate incubated at 4 °C until the viscosity was markedly reduced. The crude membrane fraction was collected by centrifugation at 356000*g* for 1 h, resuspended in a minimal volume of 50 mM sodium phosphate, pH 6.5 and 1 mM $MgCl_2$, and briefly sonicated.

Some of the crude membranes were subjected to a high-salt/urea wash. To resuspended, sonicated crude membranes were added NaCl and urea to 1 and 5 M, respectively, followed by incubation at 4 °C for 15 min and harvesting at 356000*g* for 1 h.

Crude membranes were fractionated on sucrose step-gradients (Osborn et al., 1972). Identification of the bands corresponding to inner (cytoplasmic) and outer membranes was confirmed by assaying for the relative abundance of 2-keto-3-deoxyoctonate (KDO; a unique component of lipopolysaccharide, which is located mainly in the outer membrane) (Karkhanis et al., 1978).

In Vivo Half-Life Experiments. The half-life of BO expressed in *E. coli* was determined as described (Dunn et al., 1987), with the following modifications. NCM533 containing pβgbop was grown overnight at 37 °C in SMG medium and subcultured to an OD_{600} of ~0.01 into 50 mL of prewarmed SMG medium in a 250-mL Klett flask. Growth was followed

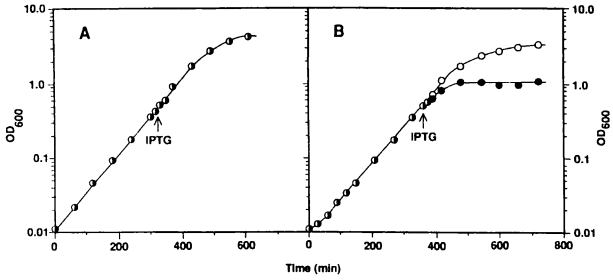

FIGURE 2: Effect of induction of vectors pEVM (A) and pβgbop (B) on growth in *E. coli* NCM533. Cultures were grown in 50 mL of SMG medium, and immediately before induction (indicated by arrows), half of the culture was transferred to a second warmed flask. Half of the divided culture was induced with 1 mM IPTG (closed circles); the other served as the uninduced control (open circles). Half-filled circles represent preinduced time points, or time points in which both induced and uninduced cultures had identical or nearly identical OD_{600} readings.

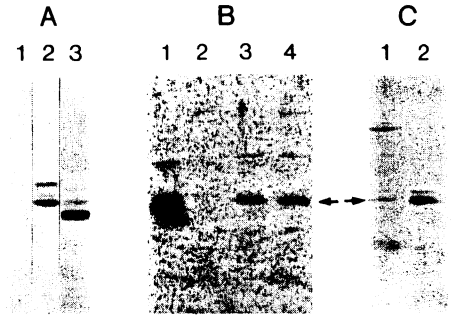

FIGURE 3: Western blot analysis of various subfractions of induced *E. coli* NCM533 containing pβgbop. (A) Comparison of uninduced (lane 1) and induced (lane 2) cultures. Equal amounts of protein (80 ng) from *E. coli* crude lysates were analyzed. Lane 3 is purple membrane. (B) Washing of total, crude membranes with urea and NaCl as described under Materials and Methods: purple membrane (lane 1), supernatant after washing (lane 2), washed membranes (lane 3), and sonicated total membrane fraction prior to washing (lane 4). Equivalent amounts of total protein (from 1 mL of cells) were assayed. (C) Sucrose gradient fractionation of total crude membranes (see Materials and Methods). Membrane components were separated and harvested from sucrose gradients. Identification of the outer and inner membrane fractions was determined by KDO assay (see Materials and Methods). The fractions were pelleted by centrifugation and resuspended in equivalent volumes of sample buffer prior to Western blot analysis. The relative proportions of e-BO associated with the outer membrane fraction (lane 1) and the inner membrane fraction (lane 2) were determined by densitometry. The typical multiband pattern of e-BO is not seen as clearly in panels B and C (arrows) as in panel A due to the lower acrylamide (12% vs 15%) concentration of the resolving gel.

by using a Klett–Summerson colorimeter (Klett Manufacturing Co., New York, NY) equipped with a blue filter. The culture was induced by adding IPTG to 1 mM at 104 Klett units (uncorrected; equivalent to an OD_{600} of ~0.45). At 30-min postinduction, the culture was pulsed with 10 mCi/mL L-[^{35}S]methionine and then chased 3 min later by adding unlabeled L-methionine to a concentration of 10 μM. Samples (1 mL) were taken at intervals (see Figure 4) starting immediately before the addition of the unlabeled methionine and placed into chilled 1.5-mL microcentrifuge tubes containing 100 μL of 11 mg/mL chloramphenicol in ethanol and then mixed. When convenient, cells were harvested by centrifugation for 5 min at 4 °C in a Fisher Model 235C microcentrifuge; the supernatant was removed and the pellet quick-frozen on dry ice/ethanol and stored at −60 °C.

The frozen pellets were thawed, solubilized, and BO-immunoprecipitated as described (Dunn et al., 1987) using 15 μL of the immune serum previously absorbed to *E. coli*. As a control, some of this immune sera was absorbed against BR by mixing equal volumes of antisera and octyl β-glucoside solubilized PM (0.58 mg/mL) and incubating overnight at 4 °C. Fifteen microliters of this mixture was used for the BR-absorbed antiserum control (see Figure 4).

Twenty microliters of the immunoprecipitated BO that had been released from the immunoabsorbent from each time point was mixed with 5 μL of 5× sample buffer (Laemmli, 1970) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). The gel was soaked in a mixture of water/acetic acid/methanol (5:4:1) for 1.5 h followed by signal enhancement in Dupont "Enlightning" (NEN Research Products, Boston, MA). The gel was dried and fluorography performed at −80 °C for 4 h on Kodak XOmat-AR film. The autoradiogram was scanned at 626 nm using a Joyce-Loebl Ephortec densitometer.

Amino-Terminal Sequencing of the Three Different Species of e-BO. The three e-BO polypeptides expressed and purified from *E. coli* containing pβgbop were separated by SDS–PAGE and electroeluted at room temperature for 4 h at 60 mA in a Bio-Rad Model 422 electroeluter using SDS–PAGE running buffer. The electroeluted samples (~2 mL) were concentrated to 0.3 mL by centrifugation in a Centricon 10 (Amicon Corp., Danvers, MA) and then applied to a 0.75 × 60 cm TSK G3000SW size-exclusion column using a 0.1% SDS/100 mM acetate, pH 6.0, mobile phase (Miercke et al., 1991). The purified proteins (0.1 mL) were directly spotted onto trifluoroacetate-treated glass fiber filter disks impregnated with BioBrene Plus (Applied Biosystems, Foster City, CA) and then subjected to an Applied Biosystems 470A gas-phase sequenator and an on-line ABI 120A phenylthiohydantoin analyzer.

Results and Discussion

Effect of Induction of Bacterioopsin on E. coli Growth Rate and Cell Viability. Figure 2 shows the effect of induction of the *bop* gene from the two vectors, pEVM and pβgbop (see Figure 1), on cell growth in *E. coli*. Addition of IPTG to an exponentially growing culture of pEVM-containing cells had no effect on growth rate as both the induced and uninduced cultures exhibited the same growth kinetics (Figure 2A). Furthermore, expression of BO from pEVM was not detectable by Western blot analysis although *bop* mRNA was detectable by Northern analysis (data not shown).

The pβgbop vector was constructed to mitigate expression difficulties by stabilizing the 5′ terminus of the *bop* mRNA and/or the amino terminus of BO expressed in *E. coli*. DNA encoding 13 heterologous residues, the first 8 of which were derived from the inducible, stable, and abundant *E. coli* cytoplasmic protein β-galactosidase, was engineered onto the 5′ terminus of the *bop* gene (Figure 1). In contrast to the results seen with pEVM, addition of IPTG to a culture of pβgbop-containing cells resulted in a significant decrease both in growth rate and in final cell density (Figure 2B). A comparison of the number of viable cells (determined by spread-plate counts) to the number of cells from direct cell counts (determined by microscopic enumeration using a Petroff–Hausser counting chamber) at 3-h postinduction revealed a 60–90% reduction in cell viability in the culture expressing BO. The expression of the fusion protein from the pβgbop vector (designated "e-BO") was detected by Western blot analysis (Figure 3A). e-BO expression levels in NCM533 were highest when the cells were grown in SMG medium, induced between OD_{600} = 0.4 and 0.8, and harvested within 45-min postinduction. Preparative-scale (12- or 100-L) cultures of NCM533 containing either pβgbop or site-directed mutations generated as described (see Materials and Methods) and expressed in the pβgbop vector maintained the same

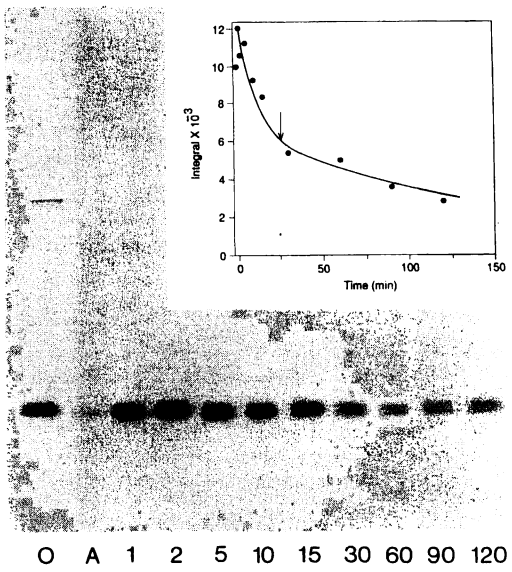

FIGURE 4: Determination of the half-life of e-BO. The autoradiogram shows ^{35}S-labeled immunoprecipitated e-BO samples after SDS–PAGE. The numbers indicate time in minutes following addition of unlabeled methionine. Lane "A" represents a sample taken 1 min after the addition of unlabeled methionine and in which e-BO was immunoprecipitated by using antibody absorbed to purple membrane (see Materials and Methods). Only two of the three e-BO bands (see Figure 3, panel A, and Figure 5) are visible on the autoradiogram due to differences in acrylamide concentration used in the resolving gel. The autoradiogram was subjected to densitometry, and a graphic representation of the data (integrated peak area versus time) is shown in the inset. The arrow indicates the half-life of e-BO.

generation time (~56 min) and the same pre- and postinduction growth kinetics as shown in Figure 2B.

Cellular Location of e-BO. e-BO was localized to the *E. coli* membrane fraction by Western blot analysis of subcellular fractions (Figure 3). The amount of e-BO in the cytoplasm was negligible. Washing of total membranes containing e-BO with 5 M urea and 1 M NaCl failed to release any of the protein (Figure 3B), indicating a firm association between the hydrophobic e-BO and the *E. coli* membranes. Subfractionation of the membranes revealed that 82% of the e-BO was associated with the *E. coli* cytoplasmic (inner) membrane and 18% with the outer membrane (Figure 3C). Dunn et al. (1987) have reported that in preliminary experiments, BO expressed from some of their vectors may be localized in the cytoplasmic membrane.

Half-Life Determination of e-BO in Vivo. [^{35}S]Methionine pulse–chase experiments (see Materials and Methods) were performed to determine the in vivo half-life of e-BO expressed from the vector pβgbop to establish when and how quickly cell cultures should be harvested and processed for protein purification. The characteristic multiband pattern of e-BO and a greatly reduced signal (Figure 4, lane A) using antiserum lacking BR-specific polyclonal antibodies indicated that the major immunoprecipitated protein detected was indeed e-BO (Figure 4). Graphic representation of the data shows first-order, biphasic decay kinetics (inset Figure 4) similar to those seen by Dunn et al. (1987). The in vivo half-life of e-BO was determined to be ~26 min which is sufficient to allow harvesting and processing of cells for purification of e-BO without extensive protein degradation. For comparison, BO containing only a single heterologous amino-terminal methionine has a reported half-life of 8–10 min (Karnik et al., 1987). Other constructions containing from as few as 7 to as many as 33 heterologous residues at the amino terminus had half-lives up to 40 min (Dunn et al., 1987; Karnik et al., 1987). Although the half-life of BO expressed from other vectors could be significantly increased by the addition of heterologous amino acids to the amino terminus, yields remained low (~0.5% of total protein; Nassal et al., 1987) regardless of the half-life.

Characterization of e-BO. The pβgbop vector construction encodes a BO fusion protein with 13 heterologous residues at the amino terminus and retains DNA encoding the carboxy-terminal aspartic acid residue (Figure 1). In comparison to BR purified from *H. halobium*, the e-BO expressed in *E. coli* from pβgbop displayed a slightly retarded electrophoretic mobility on Western blots of SDS–polyacrylamide gels (Figure 3A). Both BR and e-BO exhibited multiband patterns on Western blots (Figure 3A). In the case of BR, the three distinct bands represent various stages of in vivo amino-terminal processing of the leader sequence (Wolfer et al., 1988; Miercke et al., 1989a). In addition, the carboxy-terminal aspartic acid residue encoded in the nucleotide sequence apparently is removed from BR (Dunn et al., 1981). Bacterioopsin protein expressed in the yeast *Schizosaccharomyces pombe* (Hildebrandt et al., 1989) also exhibits a multiband pattern on Western blots, although it is not known what polypeptides the various bands represent.

The molecular mass and relative abundance of the polypeptides corresponding to the various e-BO bands are different than those seen for BR (Figure 3A). Unlike the case for BR, the three e-BO bands exhibit the same relative intensities over time and appear at the earliest detectable time (~15 min) postinduction. Densitometric analysis of e-BO on Western blots revealed that the upper and middle bands represent approximately 40% of the total, with the upper band usually being more abundant than the middle band (Figure 3A, lane 2). The lower band invariably represents 60% of the total. Attempts to inhibit or otherwise perturb processing of e-BO by addition of various protease inhibitors were unsuccessful.

To determine which end of the protein was being modified, purified e-BR incorporated into phospholipid vesicles was subjected to proteolytic cleavage by chymotrypsin (Miercke et al., 1989b) to generate C_1 (191 residues) and C_2 (71 residues) fragments (Gerber et al., 1979). The C_1 fragments from either chymotrypsin-treated e-BR or e-BR mutant proteins migrated as a single species on SDS–PAGE (data not shown), showing that the carboxy terminus was homogeneous and suggesting that modifications were occurring at the amino terminus. In addition, mass spectrometric data (A. Burlingame and A. Falick, University of California, San Francisco Mass Spectrometry Facility) show that the carboxyl-terminal aspartic acid encoded in the nucleotide sequence is present in purified e-BO.

Each species of e-BO was purified [see Materials and Methods and Miercke et al. (1991)] and subjected to Edman degradation reactions (data not shown) in order to determine the amino acid sequence at the amino terminus. The fastest migrating band lacked only the amino-terminal formylmethionine residue and had the amino acid sequence Thr-Met-Ile-Thr-Asp at the amino terminus. The eight amino-terminal residues of e-BO are identical with those of β-galactosidase (Figure 1), and processing of the amino-terminal formylmethionine from wild-type β-galactosidase occurs routinely in vivo in *E. coli* (Zabin & Fowler, 1978). Surprisingly, the middle band lacked four amino-terminal residues (fMet-Thr-Met-Ile) yet migrated slower than the e-BO species that lacked only the formylmethionine residue. The slowest migrating band, although eluted and purified in exactly the same fashion as the other two bands, was totally refractory to amino-terminal sequencing by Edman degradation. This e-BO

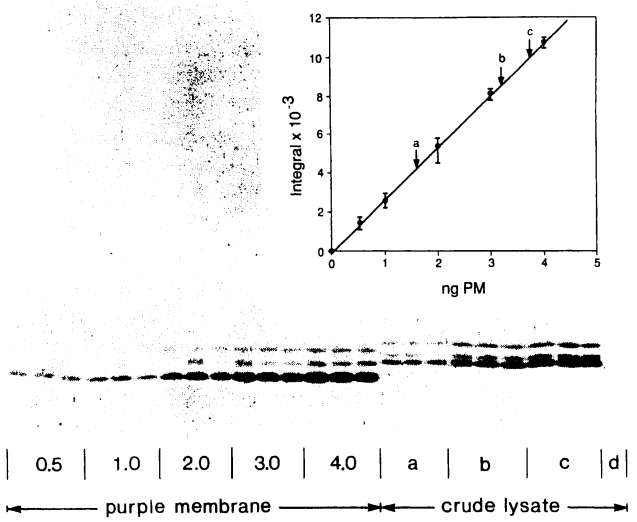

FIGURE 5: Quantitation of e-BO in an *E. coli* crude lysate by Western blot analysis. Two, 1-L cultures of NCM533 containing pβgbop were grown in parallel: one culture was induced with IPTG; the other served as an uninduced control. The cells were harvested and lysed as described in the legend to Figure 2, and the total protein (~300 mg in ~10 mL for each culture) was determined by the method of Lowry (Lowry et al., 1951) as modified by Markwell et al. (1978). The concentration of bacteriorhodopsin in purple membrane was determined spectrophotometrically by using the light-adapted molar extinction coefficient of 63000 (Miercke et al., 1989). Triplicate dilutions of purple membrane (0.5–4.0 ng) and crude lysate were subjected to Western blot analysis. 25 ng (lane a), 50 ng (lane b), and 75 ng (lane c) of protein from the induced culture and 75 ng (lane d) of protein from the uninduced culture were analyzed. The protein bands on the blot were quantitated by densitometry, and a standard curve of purple membrane concentration versus average integrated peak area was plotted (see inset; vertical bars represent range). Arrows a, b, and c in the inset indicate the average integral obtained from the 25, 50, and 75 ng of protein, respectively, from the crude lysate of the induced culture.

species probably contains the formylmethionine residue at the amino terminus, as amino-terminal formylmethionine residues are inaccessible to Edman degradation reactions (Edman, 1956).

Quantitation of e-BO. e-BO from *E. coli* crude lysates was quantitated by densitometry of Western blots (Figure 5) using various dilutions of PM to generate a standard curve (inset Figure 5). Only the linear range of the standard curve [from ~0.5 ng (the limit of detection) to ~4 ng of PM; Figure 5] was used to determine e-BO concentrations. From the data in Figure 5, e-BO constituted 5.8 ± 0.3% of the total cellular protein, which represents 17.6 ± 0.8 mg of e-BO produced per liter of cells. This yield represents a 10–20-fold increase over the highest reported levels of BO expressed in *E. coli* and is sufficient for functional analysis, the initial aspects of which are reported in Miercke et al. (1991).

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