Protein Science (1996), 5:456-467. Cambridge University Press. Printed in the USA.
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Overexpression of bacterio-opsin in *Escherichia coli* as a water-soluble fusion to maltose binding protein: Efficient regeneration of the fusion protein and selective cleavage with trypsin

GUO-QIANG CHEN AND J. ERIC GOUAUX
Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637
(RECEIVED October 25, 1995; ACCEPTED December 12, 1995)

Abstract

Bacteriorhodopsin (bR) is a light-driven proton pump from *Halobacterium salinarium* and is a model system for studying membrane protein folding, stability, function, and structure. bR is composed of bacterio-opsin (bO), the 248-amino acid apo protein, and all-*trans* retinal, which is linked to lysine 216 via a protonated Schiff base. A bO gene (sbOd) possessing 29 unique restriction sites and a carboxyl-terminal purification epitope (1D4, nine amino acids) has been designed and synthesized. Overexpression of bO was achieved by fusion to the carboxyl terminus of maltose binding protein (MBP). The expressed fusion protein (MBP-sbO-1D4) formed inclusion bodies in *Escherichia coli* and, following solubilization with urea and removal of the urea by dialysis, approximately 170 mg of ~75% pure MBP-sbO-1D4 was obtained from 1 L of culture. MBP-sbO-1D4 formed high molecular weight (≥2,000 kDa) oligomers that were water-soluble. The synthetic bO with the 1D4 tag (sbO-1D4) was separated from MBP by trypsin cleavage at the factor Xa site between the MBP and sbO-1D4 domains. Selective trypsin cleavage at the factor Xa site, instead of at the 14 other potential trypsin sites within bO, was accomplished by optimization of the digestion conditions. Both MBP-sbO-1D4 and sbO-1D4 were regenerated with all-*trans* retinal and purified to homogeneity. In general, 6-10 mg of sbR-1D4 and 52 mg of MBP-sbR-1D4 were obtained from 1 L of cell culture. No significant differences in terms of UV/vis light absorbance, light/dark adaptation, and photocycle properties were observed among sbR-1D4, MBP-sbR-1D4, and bR from *H. salinarium*.

Keywords: bacteriorhodopsin; *Escherichia coli*; maltose binding protein; membrane protein overexpression; membrane protein refolding; selective trypsin cleavage; synthetic gene

Bacteriorhodopsin (Khorana, 1993) possesses many features that render it an excellent molecule to elaborate the relationships between elements of structure – primary, secondary, and tertiary – and the function, folding, and thermodynamic properties of a polytopic integral membrane protein. bR is composed of a 248-amino acid apo protein, bacterio-opsin (Khorana et al., 1979; Ovchinnikov et al., 1979), and a polyene cofactor, all-*trans* retinal (Oesterhelt & Stoeckenius, 1971). A protonated Schiff base covalently links retinal to lysine 216 (Bayley et al., 1981). Some of the favorable properties of bR include reversible denaturation of bR to bO and retinal (Huang et al., 1981; London & Khorana, 1982), stability and function in nonnative bilayer environments and detergent micelles (Wildenauer & Khorana, 1977; Huang et al., 1980; London & Khorana, 1982; Milder et al., 1991), assembly from proteolytic or synthetic peptide fragments (Huang et al., 1981; Liao et al., 1983; Kahn & Engelman,

Reprint requests to: J. Eric Gouaux, Department of Biochemistry and Molecular Biology, The University of Chicago, 920 East 58th Street, Chicago, Illinois 60637; e-mail: gouaux@biovax.uchicago.edu.

Abbreviations: bO, wild-type bacterio-opsin from *Halobacterium salinarium*; bR, wild-type bacteriorhodopsin from *H. salinarium*; BCIP, 5-bromo-4-chloro-3-indolylphosphate; IEF, isoelectric focusing; CHAPSO, 3-[(3-cholamidopropyl)-dimethylamino]-2-hydroxyl-1-propane; CIP, calf intestinal phosphatase; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; IPTG, isopropyl-β-D-thiogalactoside; MBP, maltose binding protein; MBP-sbO, MBP fused to the amino terminus of bacterio-opsin expressed from this synthetic gene; MBP-sbO-1D4, the MBP-sbO fusion protein tagged at the carboxyl terminus with the 1D4 epitope; MBP-sbR-1D4, MBP-sbO-1D4 regenerated with all-*trans* retinal; NBT, nitroblue tetrazolium; β-NG, nonyl-β-D-glucoside; β-OG, octyl-β-D-glucoside; pEG-sbOd, derivative of the pMal-c2 plasmid with the sbOd gene fused to the 3′ end of MBP gene; PGS, PCR gene synthesis; sbO, bacterio-opsin expressed from this synthetic gene; sbO-1D4, sbO tagged with the 1D4 epitope; sbOd gene, the sbO gene with the 1D4 tag; sbR-1D4, sbO-1D4 regenerated with all-*trans* retinal.

1992), availability of a three-dimensional model (Henderson et al., 1990), extensive biophysical data (for reviews see: Mathies et al., 1991; Lanyi, 1993), and information on a vast number of site-directed mutants (Krebs & Khorana, 1993).

Detailed studies of bR necessitate efficient methods for protein overexpression and purification. Although there are many expression hosts and purification schemes for bO that involve using *Halobacterium salinarium* (Ni et al., 1990; Krebs et al., 1991; Ferrando et al., 1993), *Escherichia coli* (Braiman et al., 1987; Shand et al., 1991; Pompejus et al., 1993), yeast (Hildebrandt et al., 1991), and in vitro approaches (Bauer et al., 1992; Sonar et al., 1993), none of these systems are ideal for preparing large quantities of bO mutants that may have dramatically different solubility characteristics from the wild-type protein. Because one of our aims is to produce and characterize mutants with altered hydrophobicity, we do not want to use a system that involves insertion of bO into the membrane. Indeed, most of the existing expression schemes depend on insertion of bO into the membrane because cytoplasmic bO, which is not in inclusion bodies, is rapidly degraded (Karnik et al., 1987; Shand et al., 1991). There is an expression system in which bO is produced as inclusion bodies. However, it relies on IgA protease, an enzyme that is relatively expensive, to cleave bO from its fusion partner (Pompejus et al., 1993).

As part of our efforts directed toward elucidating molecular mechanisms to describe the structure, function, folding, and stability of bR, we have designed and synthesized genes encoding bO (sbO; Chen et al., 1994) and bO with a carboxyl-terminal binding site for the 1D4 monoclonal antibody (sbOd; 1D4 tag, nine amino acids; MacKenzie et al., 1984; Oprian et al., 1987). Both of these genes have been expressed in *E. coli* as fusions with maltose binding protein. The expression constructs and purification schemes described here have the following features: (1) the designed gene facilitates cassette mutagenesis within or between the α-helical sections; (2) the fusion is abundantly expressed as inclusion bodies that are easily purified; (3) regeneration and purification of the dual fusion protein yields large quantities of bR with MBP at the amino terminus and the 1D4 tag at the carboxyl terminus (MBP-sbR-1D4); (4) the water solubility of the fusion protein allows for efficient and selective trypsin cleavage of the fusion at the factor Xa site; (5) chromatographic purification gives homogenous refolded synthetic bR with the 1D4 tag (sbR-1D4); and (6) the 1D4 tag facilitates protein purification and may enable the crystallization of sbR-1D4 or MBP-sbR-1D4 as a complex with the Fab or Fv fragment derived from the 1D4 antibody.

Expression of hydrophobic membrane proteins as fusions with MBP followed by selective trypsin cleavage may provide a generally useful expression and purification strategy. Fusion of a hydrophilic domain to the membrane protein of interest can reduce toxicity and improve the stability of the target protein (Pompejus et al., 1993). The MBP fusions of bO with the 1D4 tag (MBP-sbO-1D4) and without it (MBP-sbO) were expressed and isolated as protease-resistant inclusion bodies. Following solubilization of the inclusion bodies with urea and removal of the urea by dialysis, the MBP fusions formed water-soluble aggregates. The MBP-sbO-1D4 fusion was regenerated with all-*trans* retinal to yield large quantities of active, monomeric protein. Application of this strategy to other membrane proteins that can be refolded from a denatured state may dramatically increase the yields of functional channels, transporters, and receptors. This is particularly important in light of the difficulty in overexpression of membrane proteins in heterologous systems (Schertler, 1992).

We have found that trypsin, a relatively nonspecific protease, selectively and efficiently cleaves sbO-1D4 from MBP-sbO-1D4 at the factor Xa site. Cleavage of sbR-1D4 from MBP is highly dependent on the concentrations of SDS and trypsin, as well as on the digestion time. These factors most likely affect the conformation of the fusion partners and the asparagine-rich linker as well as the accessibility of other potential protease sites. Although the specific conditions determined for MBP-sbO-1D4 may be unique to this system, conditions for the selective proteolysis of other membrane proteins fused to MBP may be found through systematic experimentation. Final purification of sbR-1D4 and MBP-sbR-1D4 from unregenerated and contaminating proteins, as well as from unreacted retinal, gives pigments with spectroscopic, light/dark adaptation, and photocycle properties that are not significantly different from those same properties of *H. salinarium* bR.

Results

Gene design, synthesis, and construction of pEG-sbOd

The sbO gene was designed to facilitate site-directed alterations of the amino acid sequence using cassette mutagenesis. In particular, the gene was constructed with restriction sites flanking each membrane-spanning α-helix, thus enabling the swapping of helices and loops and facilitating the assembly of complex chimeric proteins. There are 29 unique restriction sites within the sbO gene, as illustrated in Figure 1. In addition, the sequence GGAATTCCATATG containing *Eco*R I and *Nde* I sites was introduced at the 5′ end, and the sequence GGATCCAAGCTT GGCG coding for *Bam*H I and *Hin*d III sites was appended at the 3′ end of the gene. The sbO gene was synthesized by a PCR gene synthesis approach (Chen et al., 1994), thus extending existing methodology to larger genes (Prodromou & Pearl, 1992; Sandhu et al., 1992; Ye et al., 1992). A short cassette encoding the 1D4 epitope was introduced at the carboxyl terminus of the gene (MacKenzie et al., 1984; Oprian et al., 1987). The resulting construct was cloned into a MBP expression vector (pMal-c2; Maina et al., 1988).

Expression of MBP-sbO-1D4

The sbO-1D4 gene was inserted downstream from the *MalE* gene of *E. coli*, resulting in expression of the target gene product as a fusion to MBP, a water-soluble protein with molecular weight of 42.7 kDa; the molecular weight of MBP-sbO-1D4 is 68 kDa. The vector has a strong "*tac*" promoter, *MalE* translation initiation signals, and a factor Xa protease site between MBP and the target protein (Maina et al., 1988). MBP-sbO-1D4 is extremely stable in DH5α cells during expression at 37 °C. Upon induction with IPTG, the cells stopped growing while production of MBP-sbO-1D4 continued for 15 h. Approximately 170 mg of fusion protein are expressed per liter of culture.

Purification and solubility of MBP-sbO-1D4

Formation of inclusion bodies of the fusion protein minimized degradation and facilitated its purification. Water-soluble pro-

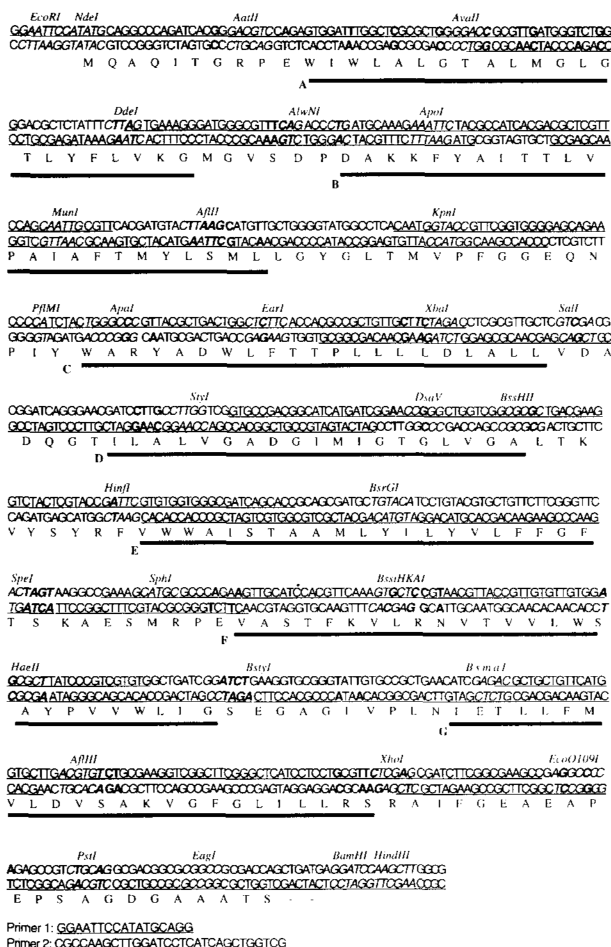

Fig. 1. DNA and amino acid sequence of the sbO gene. Underlined sequences define the 12 long oligos from which the sbO gene was synthesized, and primer 1 and 2 indicate the oligos used to amplify the gene using the PGS strategy. Nucleotides in bold represent those changed from the wild-type bO gene. Sequences in italics define the unique restriction sites, above which are the corresponding restriction enzymes. Bold lines and the letters (**A-G**) indicate the seven transmembrane sections of bO.

teins, together with some membrane proteins, were removed by centrifugation of the cell lysate and by washing the crude inclusion bodies (Fig. 2). Analysis of the cell lysate by SDS-PAGE and western blotting indicated that there were only trace quantities of MBP-sbO-1D4 that were water-soluble or that were in the cell membranes. Nevertheless, extensive washing of the inclusion bodies with Buffer 16 resulted in some loss of MBP-sbO-1D4. The material that was not solubilized by the 8 M urea/Buffer 16 solution or that precipitated following removal of the urea by dialysis contained very little MBP-sbO-1D4. The desired fusion protein comprised approximately 75% of the total protein from the solubilized and dialyzed inclusion bodies. This MBP-sbO-1D4 solution was the substrate for trypsin digestion. However, for cleavage using factor Xa, further purification of MBP-sbO-1D4 by amylose affinity chromatography was necessary.

Water solubility of the fusion protein was demonstrated by elution from the amylose column in the absence of detergent, chaotrope, or organic solvent. Buffer 4 with 10 mM maltose served to elute the fusion protein from the resin. In fact, the solution was free of turbidity and exhibited minimal light scattering at OD_{280} values as high as 2.7, as illustrated in Figure 3. Gel filtration (Fig. 4A) on Superose 6 resin in Buffer 4 showed that the fusion protein eluted in the exclusion volume, indicating that MBP-sbO-1D4 was in a highly aggregated but soluble form. Addition of SDS to the fusion protein reduced the size to approximately 170 kDa, as determined by comparison with protein molecular weight standards (Fig. 4B). The 170-kDa species might correspond to a dimeric protein–SDS complex or to an asymmetric monomeric protein–SDS complex. When analyzed by SDS-PAGE, the fusion protein migrated at a size corresponding to a monomer (see Figs. 5, 6). Tailing of the 170-kDa peak on the gel filtration column in the detergent-free buffer (Fig. 4B) may be caused by loss of SDS from the protein–detergent complex and nonspecific adhesion of the fusion protein to the resin.

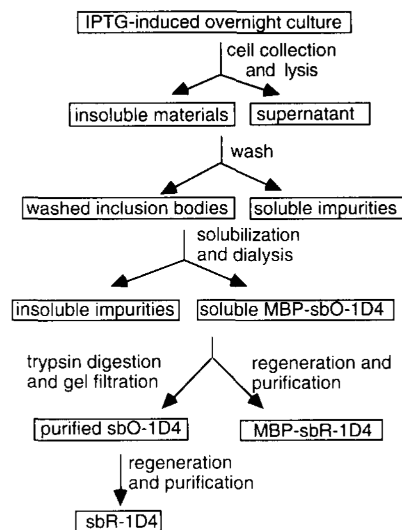

Fig. 2. Flow chart describing the steps for purification and regeneration of MBP-sbR-1D4 and sbR-1D4.

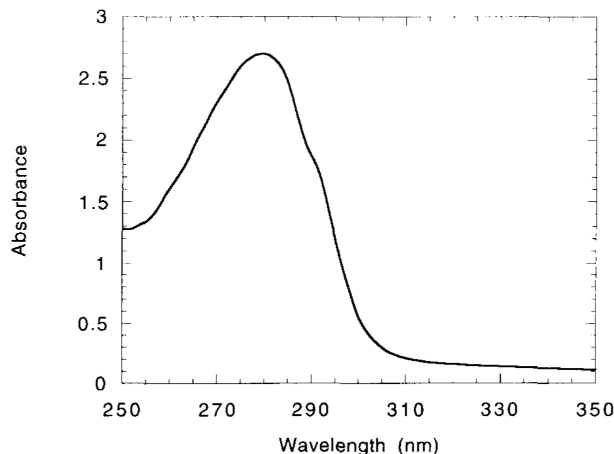

Fig. 3. UV spectrum of MBP-sbO-1D4, solubilized in aqueous solution, in the absence of detergent. The protein does not form aggregates that scatter light, even at high protein concentrations.

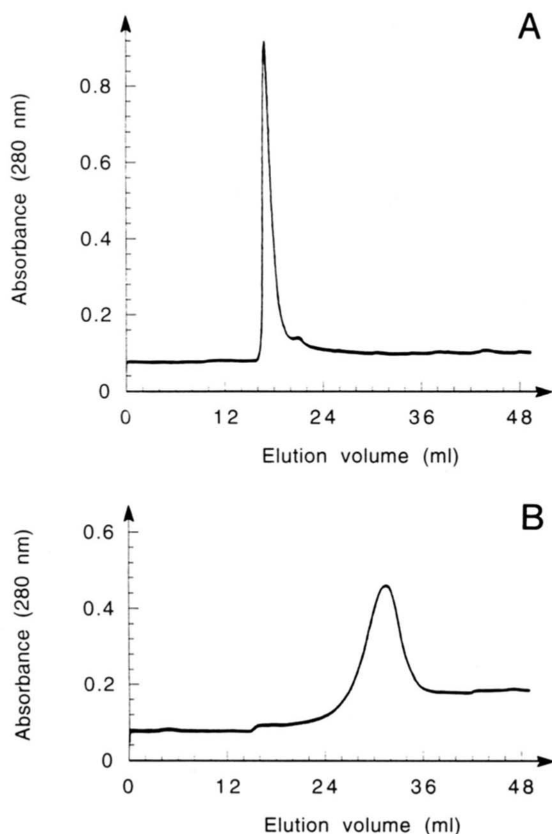

Fig. 4. Chromatographic behavior of MBP-sbO-1D4 on a Sepharose 6 column in an aqueous buffer in the absence of detergent (Buffer 4) using samples prepared without (**A**) and with (**B**) 2% SDS. The *y* ordinates give the absorbances at 280 nm in arbitrary units and the *x* ordinates show the elution volumes.

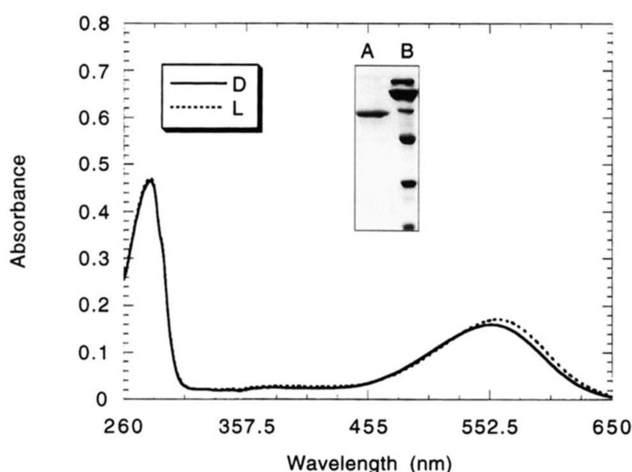

Fig. 5. UV/Vis spectra of the purified MBP-sbR-1D4 before (D) and after (L) light exposure. The peak at 554 nm (D) shifts to 559 nm (L) upon light exposure. The $A_{280}/A_{\lambda max}$ ratios are 2.86 (D) and 2.73 (L). The inset is a 12% denaturing polyacrylamide gel, stained with Coomassie Brilliant Blue, of the purified MBP-sbR-1D4 (lane A) and protein molecular weight markers (lane B: 200 kDa, 116 kDa, 97 kDa, 69 kDa, 45 kDa, 31 kDa, and 21 kDa).

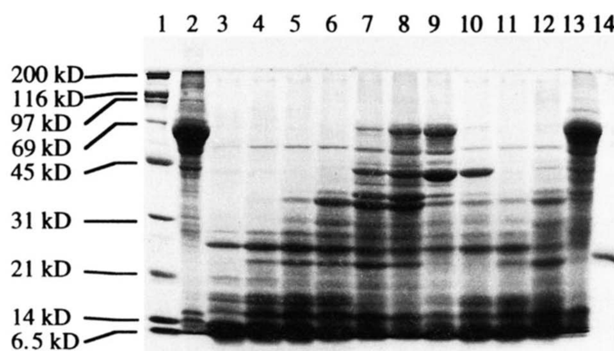

Fig. 6. Analysis of the trypsin cleavage of MBP-sbO-1D4 by SDS-PAGE and Coomassie Brilliant Blue. Lane 1, protein MW markers; lane 2, MBP-sbO-1D4; lanes 3–8, reaction mixtures with trypsin concentrations of 40, 20, 10, 5, 2, and 1 μg/mL, respectively; lanes 9–13, reaction mixtures with trypsin concentrations of 10 μg/mL and SDS concentrations of 0, 0.05%, 0.2%, 0.5%, and 1%, respectively; lane 14, bR from purple membrane.

Refolding, purification, and characterization of MBP-sbR-1D4

Addition of SDS was necessary for the refolding of MBP-sbR-1D4. Protein aggregation during reconstitution was minimized by using a low concentration of protein (0.35 mg/mL). On the basis of absorption at 554 nm, 80 mg/L of culture (45–55% yield) of the fusion protein were refolded. Four-fold concentration of the reaction mixture did not increase the ratio between the absorbance at 280 nm and the absorbance at the λ_{max} of the chromophore ($A_{280}/A_{\lambda max}$), which indicated that no aggregation or denaturation occurred. After purification by Q-sepharose and Red A chromatography, 52 mg of purified MBP-sbR-1D4 were obtained. Shown in Figure 5 are the UV/vis spectra of the purified MBP-sbR-1D4 under dark (D) and light (L) adaptation; analysis of the purified protein by SDS-PAGE is also presented. Light exposure of the dark-adapted protein resulted in a red shift of the chromophore from 554 nm to 559 nm. The $A_{280}/A_{\lambda max}$ ratio was 2.86 for the dark-adapted form and 2.73 for the light-adapted form. Gel filtration analysis of the purified MBP-sbR-1D4 in Buffer 14 showed that its molecular weight was 92 kDa, an indication that the refolded MBP-sbR-1D4 existed as monomers in β-OG micelles.

Factor Xa cleavage of MBP-sbO

The factor Xa protease site (Ile-Glu-Gly-Arg) is located between the carboxyl terminus of MBP and the amino terminus of sbO and is preceded by a potentially flexible and solvent-exposed linker of 10 asparagine residues. Nonetheless, in the absence of SDS, factor Xa does not cleave the fusion protein purified by affinity chromatography. Systematic experiments showed that a low concentration of SDS (0.05%, 1.7 mM) was optimal. However, factor Xa cut not only the desired site, but also the sites within the fusion partners, even under the most favorable conditions, as shown by SDS-PAGE using Coomassie staining and western blotting. This poor selectivity resulted in a low yield of sbO (estimated at <30% by SDS-PAGE). Digestion of MBP-sbO that was not purified by affinity chromatography resulted

in an even lower yield of sbO and produced numerous fragments that were difficult to separate from the full-length protein.

Highly selective trypsin cleavage of MBP-sbO-1D4

To selectively and efficiently cleave the fusion protein with trypsin, it was necessary to carefully optimize the concentrations of SDS and trypsin, as illustrated in Figure 6. The best results were achieved with 0.2–0.4% SDS and a 350–700:1 ratio (wt:wt) of fusion protein to trypsin. In addition, the reaction time was another important factor. We found that a higher concentration of trypsin coupled with a shorter reaction time (1–2 h) gave a higher yield and more selective cleavage than a lower concentration of trypsin and a longer time. Simplification of the purification could be achieved by allowing the digestion to go slightly longer than estimated as optimal on the basis of small-scale reactions analyzed by SDS-PAGE. Moderate overdigestion gave fragments that were easier to purify away from sbO-1D4 than the fragments from underdigestion. The yield of sbO-1D4 from MBP-sbO-1D4 was about 30–40% on the basis of a number of sbO-1D4 preparations. Amino acid sequencing clearly showed the sequence of the first 15 residues downstream of the factor Xa site (ISEFHMQAQITGRPE), indicating that trypsin selectively cleaved at the desired factor Xa site.

Purification of sbO-1D4

From the optimized trypsin reaction, the major protein was sbO-1D4, combined with other mostly smaller fragments (Fig. 7, lane 6). The sbO-1D4 obtained from the trypsin digestion was purified to 90–95% using a 1-meter Superose 12 gel-filtration column. The purity of sbO-1D4 was estimated from SDS-PAGE (Fig. 7, lane 7) and amino acid sequencing. Further purification by a wide range of different resins under many conditions gave poor recoveries and did not increase homogeneity, possibly due to microheterogeneity in SDS binding and in the protein conformation. Therefore, further purification was carried out after regeneration with all-*trans* retinal. The typical yield of purified sbO-1D4 from the Superose column was about 17–26 mg/L of culture.

Refolding and purification of sbR-1D4

An increase in the absorbance at 554 nm was observed as soon as all-*trans* retinal was added to purified sbO-1D4 in Buffer 18, thus indicating correct refolding of the protein. A continuous increase in the peak intensity at 554 nm could be observed during the first few minutes following addition of retinal. This increase was essentially complete in 20 min and there was only a further small increase after overnight incubation at room temperature in the dark. Q-Sepharose column chromatography removed most of the free retinal and contaminating proteins.

Preparation and use of the 1D4 immunoaffinity resin

According to the amount of rhodopsin eluted from the 1D4-Sepharose resin, the capacity of the resin was ~1 mg/mL. For the purification of sbR-1D4, all-*trans* retinal was cleanly removed by extensive washing of sbR-1D4 bound to 1D4-Sepharose and the protein was released from the column by elution with a buffer containing the 1′-9′ rhodopsin peptide.

Even though both Q-Sepharose and 1D4-Sepharose could be used to remove excess all-*trans* retinal and polymerized proteins from the reconstitution mixture, the strategy employing the Q-Sepharose column is more practical for large-scale preparations, not only because it is commercially available, but also because the resin is more robust. Following Q-Sepharose chromatography, the $A_{280}/A_{\lambda max}$ ratio was typically 2.0, indicating that some unrefolded sbO-1D4 was present. The unrefolded sbO-1D4 was removed by a Red A affinity column (Miercke et al., 1991) and the resulting sbR-1D4 solution had a $A_{280}/A_{\lambda max}$ ratio of 1.5 (Fig. 8). In general, the final yield of purified sbR-1D4 was about 6–10 mg/L of culture.

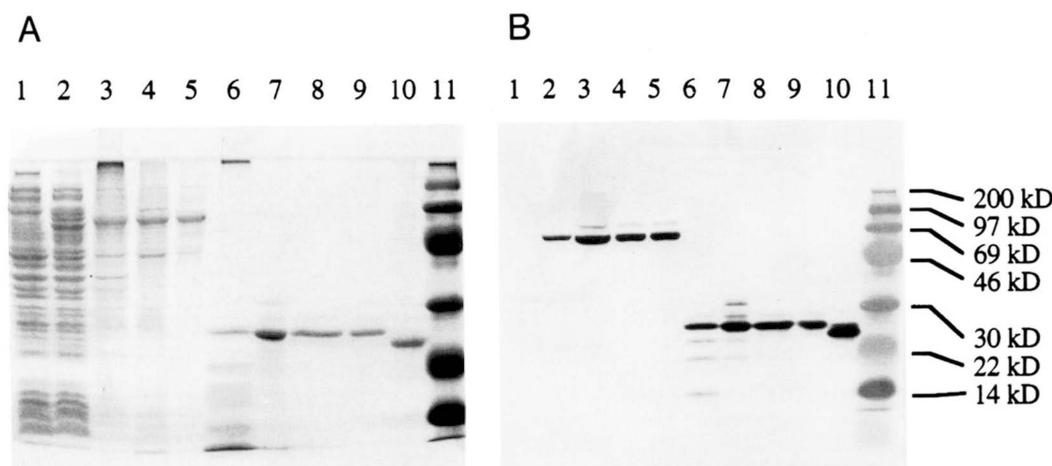

Fig. 7. SDS-PAGE of protein samples from whole cells to purified sbR-1D4. **A:** Coomassie Brilliant Blue staining. **B:** Western blot using a bR C-terminal antibody. Lane 1, uninduced cells; lane 2, IPTG-induced cells; lane 3, washed inclusion bodies; lane 4, dissolved inclusion bodies; lane 5, MBP-sbO-1D4 purified by amylose affinity column; lane 6, trypsin digestion; lane 7, sbO-1D4 purified by the Superose 12 column; lane 8, sbR-1D4 purified by the Q-Sepharose column; lane 9, sbR-1D4 purified by the Red A column; lane 10, bR from purple membrane; lane 11, protein molecular weight markers.

Homogeneity of synthetic sbR-1D4

The homogeneity of sbR-1D4 in terms of conformation, size, and charge was determined. The $A_{280}/A_{\lambda max}$ ratio indicated that sbR-1D4 is not significantly contaminated with other proteins, but also that essentially all of the protein is in the active, retinal-bound conformation. Gel-filtration chromatography of the purified sbR-1D4 in Buffer 14 gave a single peak with an estimated molecular weight of 50 kDa, corresponding to a sbR-1D4 monomer in a β-OG micelle (Muccio & DeLucas, 1985). This indicated that the protein is not aggregated under nondenaturing conditions. Isoelectric focusing showed that the sbR-1D4 had two major bands at pI 5.56 and pI 5.33, compared to bR from purple membrane, which gave multiple bands between pI 5.9 and 5.1. More than 95% of the total synthetic protein was focused at these two bands: approximately 75% was at pI 5.56 and 25% focused at pI 5.33. Existence of the lower pI (5.33) protein may be due to hydrolysis of asparagine to aspartic acid, for example.

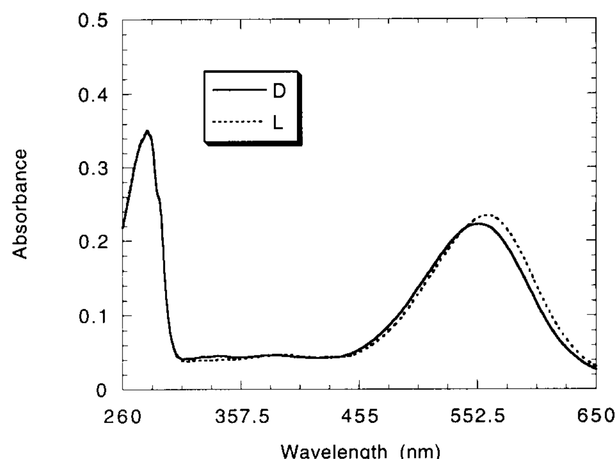

Fig. 8. Purified sbR-1D4 in β-nonyl glucoside before (D) and after (L) light exposure. The peak at 554 nm (D) shifts to 561 nm (L) upon light exposure. The $A_{280}/A_{\lambda max}$ ratios are 1.57 (D) and 1.50 (L).

Activity of MBP-sbR-1D4 and sbR-1D4

Listed in Table 1 are the λ_{max} values and the $A_{280}/A_{\lambda max}$ ratios of the dark- and light-adapted forms of sbR-1D4, MBP-sbR-1D4, and bR from *H. salinarium*. Photocycle experiments showed that detergent-solubilized MBP-sbR-1D4 and sbR-1D4 gave characteristic M and O intermediates with absorbance maximum and lifetimes similar to the wild-type bR isolated from *H. salinarium*: M (λ_{max} = 410 nm) and O (λ_{max} = 640 nm) (Krebs & Khorana, 1993).

Discussion

To a large degree, elucidation of structure and function relationships in membrane proteins is hampered by difficulties in overexpression in *E. coli* and in other systems. One reason may be due to toxicity, which can result from insertion of large quantities of protein into the membrane. A second cause may be degradation of protein that has not been inserted into the membrane (Grisshammer et al., 1993) or that is not in inclusion bodies. A third factor may simply involve the smaller volume available in the cell membrane in comparison to the cytoplasm. In fact, the half-life of bO in the cytoplasm of *E. coli* is short, only 8–10 min (Karnik et al., 1987). During our efforts to overexpress sbO, we tested pET15b and pET-21a(+) vectors combined with JM109, BL21(DE3), and BL21(DE3)LysS strains of *E. coli*. The expression levels, as monitored by western blotting using amino and carboxyl-terminal antibodies, were either undetectable or very low. For example, the expression of sbO from a pET-21a(+) vector harboring the sbO gene was low and appeared to be due, at least in part, to degradation during induction and following cell lysis.

In striking contrast, sbO fused to MBP formed inclusion bodies that were stable throughout expression and cell lysis. The inclusion bodies were isolated by a low-speed centrifugation step and assay of the remaining supernatant by western blotting showed only trace amounts of fusion protein. Because the cell membranes and any water-soluble fusion protein would remain in the supernatant, the very small amount of material detected in the supernatant indicated that only trace quantities of the fusion protein were inserted into the membrane or present in a water-soluble form. Although this result may be due to proteolytic removal of the epitope for the carboxyl-terminal antibody employed in the western blotting, staining by Coomassie did not reveal fusion protein in the supernatant either.

pMal vectors have been used commonly for expression of soluble proteins or soluble domains of membrane proteins (Ko

Table 1. *Spectroscopic properties of synthetic and native bacteriorhodopsins*

Protein	Dark-adapted λ_{max} (nm)[a]	Dark-adapted $A_{280}/A_{\lambda max}$[b]	Light-adapted λ_{max} (nm)[a]	Light-adapted $A_{280}/A_{\lambda max}$[b]	λ_{max} of protein (nm)[a]
sbR[c]	555.1	1.64	561.2	1.58	280.5
sbR-1D4	554.2	1.57	561.2	1.50	280.5
MBP-sbR-1D4	553.7	2.86	559.1	2.73	281.0
bR from purple membrane	555.1	1.52	560.0	1.49	280.6

[a] Experimental error in wavelength measurement is estimated to be 0.5–1.0 nm.
[b] $A_{280}/A_{\lambda max}$ values represent the ratios between the absorbance of λ_{max} of protein and the chromophore.
[c] G.-Q. Chen and J.E. Gouaux (unpubl. results).

et al., 1993; Hiraoka et al., 1994; Gale & Schimmel, 1995). In the cases of fusions to hydrophilic domains, the proteins are usually expressed in a soluble form. The vector pMal-p2, which possesses a periplasmic localization signal, significantly increases the expression level of an intact, G-protein-coupled receptor in comparison to a number of other common constructs, although the quantity of receptor is still quite low (Grisshammer et al., 1993). MBP-sbO-1D4 does not have a periplasmic localization signal and is not inserted into the membrane. This behavior, combined with the hydrophobicity of the sbO domain, may give rise to inclusion body formation. Therefore, one may boost expression levels of a membrane protein by specifically not targeting the protein for insertion into the membrane, thus simultaneously (1) taking advantage of the larger volume of the cytoplasm compared to the cell membrane, (2) minimizing toxicity by reducing perturbation of the cell membrane, and (3) reducing degradation through the formation of insoluble, protease-resistant inclusion bodies. Because the extant *E. coli* expression and purification schemes for bR do not give the overall yield and purity of regenerated and purified bR (Braiman et al., 1987; Miercke et al., 1991; Pompejus et al., 1993), it is difficult for us to make direct, quantitative comparisons to the other work. Nonetheless, reasonable estimations indicate that the expression and purification schemes described here give larger quantities of sbR-1D4 ($\geq$98% purity), either alone or in a fusion with MBP, in comparison to the existing schemes.

Interestingly, MBP-sbO-1D4 that was expressed in an insoluble form in DH5α cells became water-soluble after solubilization of the inclusion bodies. Gel filtration showed that the soluble MBP-sbO-1D4 existed as high molecular weight oligomers. The hydrophobic sbO domain of the fusion protein is probably buried in the interior of the aggregate, thus giving rise to a large protein micelle. Formation and decomposition of the soluble aggregate appears to be controlled kinetically. Both the insoluble inclusion bodies and the soluble aggregate were stable over long periods of time. Of course, the insolubility of the inclusion bodies may also arise from other molecules that were removed following solubilization and dialysis. Dilution of the soluble MBP-sbO-1D4 solution from 1.2 mg/mL to 0.05 mg/mL did not cause dissociation of the aggregate. The water-soluble MBP-sbO-1D4 aggregate was also stable to repeated freezing and thawing and did not aggregate further or form precipitate, even at concentrations as high as 7 mg/mL. However, heating of the water-soluble aggregate produced a large quantity of precipitation. One interpretation of this observation is that thermally induced dissociation of the complex produced smaller, more hydrophobic species that were not sufficiently polar to remain in solution.

Once MBP-sbO-1D4 was refolded and purified to yield MBP-sbR-1D4, the fusion protein remained monomeric in the presence of a suitable detergent, such as β-OG. The detergent was necessary to maintain this monomeric state and dilution of the detergent below the critical micellar concentration did not result in formation of a water-soluble protein micelle, as was the case for the unrefolded, apo protein. For example, dilution or dialysis of MBP-sbR-1D4 solubilized with Buffer 14 by detergent-free Buffer 14 resulted in denaturation and precipitation of the fusion protein.

The sbO-1D4 portion in the soluble MBP-sbO-1D4 aggregate could not be regenerated with all-*trans* retinal in the absence of SDS, even when other detergents and lipids such as DMPC and CHAPSO were present. This may be because the sbO-1D4 portion in the aggregate was buried and inaccessible to retinal or because it was trapped in a kinetically inert, misfolded conformation. In addition to its crucial and well-characterized role in promoting the folding of isolated bR (Huang et al., 1981), SDS may facilitate regeneration of MBP-sbO-1D4 by dissociating the water-soluble protein aggregate so that the sbO-1D4 portion is not trapped in a nonproductive conformation. However, low concentrations of SDS were not required to maintain the conformation of the regenerated MBP-sbR-1D4. Removal of residual SDS and purification of the regenerated fusion protein to homogeneity was achieved by Q-sepharose and Red A column chromatography.

Bacteriorhodopsin expressed in *E. coli* from our synthetic gene lacking the carboxyl terminal 1D4 tag (sbR) has a λ_{max} of 555 nm (G.-Q. Chen & J.E. Gouaux, unpubl. results), which is identical to the value from *H. salinarium* bR, prepared under similar conditions. The small difference (~1 nm) in λ_{max} of sbR-1D4 compared to bR from *H. salinarium* may be due to addition of the 1D4 tag or to the experimental error of spectral measurements. As judged by the similarity between the λ_{max} of the chromophore in MBP-sbR-1D4 and in bR from *H. salinarium*, as well as by the red shift upon light adaptation, the structure and chemical environments of retinal are very similar. The purity of MBP-sbR-1D4 was determined by SDS-PAGE and by comparing the experimentally determined value of $A_{280}/A_{\lambda max}$ to the theoretical minimum $A_{280}/A_{\lambda max}$ value of completely refolded MBP-sbR-1D4, estimated by using the extinction coefficients of isolated MBP and monomeric bR (MBP, ϵ at 280 nm: 1.47 mL/mg, Kellermann & Ferenci, 1982; bR, ϵ at 555 nm: 50,900 M^{-1} cm^{-1}, Rehorek & Heyn, 1979). This gives a calculated $A_{280}/A_{\lambda max}$ ratio of completely refolded MBP-sbR-1D4 of 2.8, which is the same ratio obtained from the purified MBP-sbR-1D4, within experimental error. Although this correspondence may be fortuitous, it nonetheless indicates that the purified MBP-sbR-1D4 is essentially free from contaminating proteins, unregenerated material, and excess retinal.

Even though fusion proteins can yield large quantities of material, separation of the target protein from the fusion partner by chemical or enzymatic means often presents a major hurdle. When considering proteolytic methods, poor results may be due to inaccessibility of the protease site, to an unfavorable conformation at or near the scissile bond, or to the presence of additional, undesired reactive sites. For example, the "normal" factor Xa site is a four-amino acid sequence (Ile-Glu-Gly-Arg), but sequences like Cys-Asn-Gly-Arg (Nambiar et al., 1987) and Ser-Leu-Ser-Arg-Met-Thr-Pro (Quinlan et al., 1989) can also be recognized and cut by this protease. Our poor results in using factor Xa to cleave MBP-sbO-1D4, combined with its high cost, led us to explore using trypsin instead. Following careful optimization of the reaction conditions, we found that the partially purified MBP-sbO and MBP-sbO-1D4 were good substrates for trypsin digestion. This approach was not only much more economical, but trypsin also digested impurities in the fusion protein sample.

It is noteworthy that there are 14 sites (Arg and Lys) within sbO-1D4 that could be cut by trypsin. If there is no selectivity among different sites, then the probability of cleavage that gives intact sbO-1D4 molecules is less than $1/15^n$ after n times of cleavage. Furthermore, the value of $1/15^n$ could approach zero because of the catalytic efficiency of trypsin. However, the rou-

tine yield of 30–40% for MBP-sbO-1D4 indicated that the reaction conditions were highly selective for cleavage at the desired site. This may partially be due to binding of detergent to sbO-1D4, because the hydrophobic sbO-1D4 undoubtedly has a stronger tendency to bind SDS molecules than the hydrophilic MBP. Binding of SDS may not only change the conformation of sbO-1D4 so that it is more protease resistant, but the charged SDS head groups may also diminish the accessibility of trypsin to potential cleavage sites within sbO-1D4. It is possible that selective trypsin digestion, modulated by low concentrations of detergent or other amphiphilic reagents, may be applied to the proteolysis of other membrane protein fusion systems that contain factor Xa or related sites.

In conclusion, the very hydrophobic, toxic, and unstable bO became very stable as a fusion to MBP in *E. coli*, resulting in overexpression. The water solubility of the fusion protein and the significant difference in hydrophobicity between the two fusion partners may be responsible for efficient and highly selective cleavage by trypsin at a factor Xa site. With the *de novo* designed gene, abundant expression in *E. coli*, efficient and selective trypsin cleavage of the MBP fusion protein, and the purification strategies outlined here, we have an efficient and economical way to make bO mutants in *E. coli*. Some of the methods presented here may also facilitate the overexpression and purification of other membrane proteins. Together with the ability to refold this integral membrane protein fusion, purification to homogeneity in large quantities, and the potential application of the 1D4 tag, we have established a good system to perform further studies of membrane protein folding, stability, function, and structure using bacteriorhodopsin.

Materials and methods

Biochemicals, molecular markers, and detergents

2-Deoxyribonucleoside-5′-triphosphates, lambda DNA-*Hind* III and ϕX174 RF DNA-*Hae* III digest markers were from New England Biolabs (Beverly, Massachusetts). ATP and low melting point agarose were from GIBCO BRL Life Tech (Grand Island, New York). IPTG was from Calbiochem (San Diego, California). Sorbitol, all-*trans* retinal, blue dextran, protein gel filtration molecular weight markers [apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa)], protein IEF markers (pI 3.6–9.3), carbenicillin, Triton X-100, and reduced Triton X-100 were from Sigma (St. Louis, Missouri). All-*trans* retinal stock solutions (10 mM) were prepared in ethanol and stored at −20 °C in the dark. Protein SDS-PAGE molecular weight markers, NBT, BCIP, and Tween-20 were from Bio-Rad (Hercules, California). SDS was from J.T. Baker Inc. (Phillipsberg, New Jersey). β-OG, β-NG, and CHAPSO were from Anatrace (Maumee, Ohio). DMPC was from AVANTI Polar Lipids, Inc. (Alabaster, Alabama).

Oligonucleotides

The 2-deoxyribose oligonucleotides (oligos) were purchased from OPERON (Alameda, California) or were synthesized by the oligonucleotide facility at the Howard Hughes Institute (University of Chicago). The long oligos were purified by 12% preparative denaturing PAGE (Ausubel et al., 1993). The oligo bands were visualized by UV light shadowing using PEI-Cellulose F plates (EM Science, Gibbstown, New Jersey). The band of the desired product was cut from the gel and minced. Oligos were electroeluted from the gel slices into a dialysis bag (MWCO 3500) in Buffer 1. The eluted oligos were extracted with an equal volume of chloroform to remove organic impurities and then 0.1 volumes of 3 M sodium acetate, pH 5.0, and 2.5 volumes of 95% ethanol were added. The mixture was cooled to −20 °C overnight. The precipitate was collected by centrifugation (13,000 × *g*, 5 min) and dried *in vacuo*. Dried oligos were dissolved in Buffer 2 and the concentrations were determined spectrophotometrically (1 $OD_{260} \approx 33$ μg/mL). The short primers were used without purification.

Enzymes

Deep Vent (exo^-) DNA polymerase, restriction endonucleases, T4 ligase, CIP, factor Xa, and polynucleotide kinase were purchased from New England Biolabs. Trypsin, DNase I, and lysozyme were from Sigma. Lysozyme (10 mg/mL) and DNase I stock solutions (1 mg/mL) were aliquoted in Buffer 3. Trypsin stock solutions (1 mg/mL) were prepared in aliquots of Buffer 4 supplemented with 20 mM $CaCl_2$. All enzyme stock solutions were stored at −20 °C and used only once after thawing.

Antibodies

The carboxyl- and amino-terminal bO antibodies (Yamaguchi et al., 1993), a generous gift from Dr. Koichi Koyama (Fuji Photo Film Co., Japan), were dissolved in water and stored in aliquots at −20 °C. Goat anti-mouse IgG antibody conjugated to alkaline phosphatase was from Bio-Rad. Hybridoma cells expressing the 1D4 rhodopsin monoclonal antibody (1D4 mAb) were a gift of Dr. Robert Molday (The University of British Columbia, Vancouver, Canada). The peptide that is the 1D4 mAb epitope (rhodopsin amino acids 1′-9′; TETSQVAPA) was synthesized and purified by HPLC in the Tager laboratory (The University of Chicago).

Plasmids and E. coli strains

pUC19 and pMal-c2 vectors were purchased from New England Biolabs. Plasmids were purified by QIAprep-spin kits (QIAGEN, Chatsworth, California). DNA from 5 mL of overnight culture was eluted from the spin column with 100 μL of 10 mM Tris-HCl buffer, pH 8.0, sterilized. *E. coli* strain DH5α [genotype: F^- ϕ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169*deo*R *rec*A1*end*A1*hsd*R17(r_k^-,m_k^+)*sup*E44λ^-*thi*-1 *gyr*A96 *rel*A1] was used for both cloning and expression.

Buffers

Buffer 1: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0; Buffer 2: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0; Buffer 3: 50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0; Buffer 4: 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4; Buffer 5: 10 mM Tris-HCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, pH 7.5; Buffer 6: 0.1 M $NaCO_3$, 0.5 M NaCl, pH 8.3; Buffer 7: 10 mM sodium phosphate, 150 mM NaCl, 10 mM glycine, pH 7.0; Buffer 8: 10 mM sodium phosphate, 150 mM NaCl, 10 mM NaN_3, pH 7.0; Buffer 9: 20 mM PIPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 150 mM NaCl, 10 mM CHAPS, 2 mM DTT, pH 6.5;

Buffer 10: 25 mM Tris, 192 mM glycine, 20% methanol; Buffer 11: 20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20, pH 7.6; Buffer 12: 20 mM Tris-HCl, 137 mM NaCl, pH 7.6; Buffer 13: 0.1 M Tris, 0.5 mM $MgCl_2$, pH 9.5; Buffer 14: 400 mM sodium phosphate, pH 5.6, 0.8% β-OG; Buffer 15: 10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, 10% methanol, pH 11; Buffer 16: 50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, pH 8.0; Buffer 17: 100 mM sodium acetate, 0.1% SDS, pH 6.0; Buffer 18: 30 mM sodium acetate, 0.03% SDS, 0.5% CHAPSO, 0.5% DMPC, pH 5.0; Buffer 19: 10 mM sodium phosphate, 0.1% reduced Triton X-100, pH 5.6; Buffer 20: 10 mM sodium phosphate, 2 M NaCl, 1% β-OG, pH 5.6; Buffer 21: 10 mM sodium phosphate, 1% β-OG, pH 5.6; Buffer 22: 5 mM sodium acetate, 18 mM β-NG, pH 5.0; Buffer 23: 5 mM sodium acetate, 18 mM β-NG, 500 mM NaCl, pH 5.0; Buffer 24: 20 mM PIPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 150 mM NaCl, 10 mM CHAPS, pH 6.5. Buffer 25: 30 mM sodium phosphate, pH 5.6, 0.5% DMPC, 0.5% CHAPSO, 0.2% SDS, 5 mM maltose. Buffer 26: 10 mM sodium acetate, 500 mM NaCl, pH 6.0, 18 mM β-NG; Buffer 27: 20 mM sodium acetate, 100 mM NaCl, pH 5.0, 18 mM β-NG.

Gene design and synthesis

The sbO gene was designed using the GCG program (Devereux et al., 1984) and synthesized using a PGS approach (Prodromou & Pearl, 1992; Sandhu et al., 1992; Ye et al., 1992; Chen et al., 1994). The sequences of the 12 long oligos and 2 short oligos employed in the synthesis and amplification of the full-length sbO gene are underlined in Figure 1. Codon changes from the wild-type gene and locations of unique restriction sites are also shown in Figure 1. Details of the gene synthesis procedure have been published previously (Chen et al., 1994).

Recombinant DNA techniques

DH5α competent cells were prepared according to standard methods (Ausubel et al., 1993). Cells were grown in LB media (liquid media: 1% bacto-tryptone, 0.5% yeast extract, and 1% NaCl; agar plates were supplemented with 1.5% bacto-agar). Carbenicillin (50 μg/mL) was used when screening was necessary. Vectors cut with restriction enzymes were treated with CIP and purified on 1% agarose gels in Buffer 1. Phosphorylation of the DNA containing the 1D4 epitope coding sequence was performed as follows. The two purified single DNA strands were added to a 50-μL reaction mixture (final concentration 5 μM) containing 1× New England Biolabs buffer for T4 polynucleotide kinase supplemented with 1 mM ATP. The reaction mixture was heated to 100 °C (2 min) followed by incubation at 37 °C (15 min). Subsequently, 10 U of T4 polynucleotide kinase was added and the mixture was incubated at 37 °C (2 h). Inactivation of the kinase was accomplished by heating the reaction at 75 °C for 10 min. The insert from the phosphorylation reaction was diluted to a concentration of 5 nM and used in ligation reactions. All other inserts from restriction endonuclease digestions were gel purified (2% agarose, Buffer 1).

Ligation reactions in which the DNA remained in the agarose gel were carried out at room temperature (18–20 °C) overnight. The concentration of DNA in gel bands was estimated either by the amount of DNA used in the digestion reaction or by comparing the band intensity to DNA standards. Transformation of *E. coli* with the product of a ligation reaction was performed according to standard methods (Ausubel et al., 1993) with minor modifications. Specifically, after denaturing the ligase by brief heating to 70 °C, the liquefied mixture was added to 100 μL of Buffer 5. This mixture was used to transform 250 μL of competent DH5α cells.

The sbO gene was cloned into pUC19 at the *Eco*R I and *Bam*H I sites. Verification of the correct sequence was determined by sequencing both strands. For expression, the sbO gene was subcloned into the pMal-c2 vector using *Eco*R I and *Hin*d III sites. After the protein expression was confirmed by western blotting using the C-terminal antibody (Yamaguchi et al., 1993), a DNA fragment encoding the 1D4 epitope (bold) GGCC GCG**ACTGAAACTTCTCAGGTTGCACCGGCT***TGATGA*C and two stop codons (italics) was cloned into the 3′ end of the sbO gene at the Eag I site. The pMal-c2 expression plasmid containing the sbO gene and the 1D4 binding site (sbOd) is called pEG-sbod.

Plasmid DNA for DNA sequencing was purified by ethanol precipitation and the concentration was determined spectrophotometrically (1 $OD_{260} \approx 50$ μg/mL). DNA sequencing was carried out using the Taq DyeDeoxy Terminator Cycle sequencing Kit (Applied Biosystems). In general, 1 μg of plasmid and a final primer concentration of 200 nM were used for each 20-μL PCR reaction. The reaction mixture was covered with 1 drop of mineral oil (Sigma) and the following PCR program was performed: 96 °C (30 s)/50 °C (15 s)/60 °C (4 min) for 25 cycles. The PCR products were purified with small spin columns filled with 45 mg of Sephadex G-50 (Sigma). The eluted DNA was dried in vacuo and analyzed using an automated sequencer (Applied Biosystems).

Preparation and binding capacity of 1D4-Sepharose

CNBr-activated Sepharose 4B (2.5 gm) was suspended in 40 mL of 1 mM HCl at 4 °C and the resin was washed with 1 mM HCl (40 mL, 6 times) and kept on ice. Prior to the coupling reaction, the resin was washed with cold Buffer 6 (40 mL, two times). To couple the antibody to the resin, 60 mg of protein A-purified 1D4 in Buffer 6 was combined with the washed resin and gently mixed overnight at 4 °C. Any remaining active sites were blocked with 40 mL of Buffer 7 (room temperature, 1 h). The resin was washed with Buffer 7 and stored in Buffer 8 at 4 °C.

Bovine rhodopsin purified by Concanavalin A-Sepharose (De Grip, 1982) was used to test the binding capacity of the 1D4-Sepharose resin. To 0.30 mL of 1D4-Sepharose washed with Buffer 9 (1 mL, two times) was added 1.05 mg of purified rhodopsin in 7 mL of Buffer 9. This slurry was incubated on a Nutator overnight at 4 °C, in the dark. After the resin was separated from unbound rhodopsin by centrifugation, it was washed with Buffer 9 (1 mL, three times). The bound rhodopsin was eluted with Buffer 9 containing 35 μg/mL of the 1′-9′ rhodopsin peptide (1 mL, three times; Oprian et al., 1987). The three elutes were pooled and the amount of rhodopsin eluted from the 1D4-Sepharose resin was determined spectrophotometrically.

SDS-PAGE, western blotting, IEF electrophoresis, and analytical gel filtration chromatography

SDS-PAGE was performed using 12% resolving gels and 5% stacking gels (70 mm × 100 mm × 2 mm) (Sambrook et al.,

1989). Gels were initially run at 60 V and then at 90 V once the dye entered the resolving gel. Protein bands were visualized with Coomassie Brilliant Blue and dried between two cellophane membranes (Bio-Rad).

Western blotting experiments were carried out on nitrocellulose membranes (Amersham, Arlington Heights, Illinois). After SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane (Buffer 10, 12 V for 40 min) and the membrane was rinsed with Buffer 11 and blocked with 5% nonfat milk protein (Bio-Rad) for 60 min. The blocked membrane was rinsed twice and washed three times with Buffer 11. Each washing was carried out by gently rocking the membrane in about 100 mL of the wash buffer for 5 min. The membrane was incubated with the C-terminal antibody (Yamaguchi et al., 1993) in 20 mL of Buffer 11 (1:1,000 dilution) for 60 min and then rinsed and washed with Buffer 11 as above. The membrane was next incubated with the goat anti-mouse IgG-alkaline phosphatase conjugate in Buffer 11 (1:1,500 dilution) for 60 min. After being rinsed and washed with Buffer 11, the membrane was washed once with Buffer 12. Bands corresponding to protein were visualized in about 2 min upon incubation with 0.03% NBT and 0.015% BCIP in Buffer 13. The membrane was then rinsed with distilled water and dried between two cellophane membranes in the dark.

IEF was carried out using a 2117 Multiphor II Electrophoresis Unit (LKB) at 12 °C under dim light. Agarose gels (1%) containing 12% sorbitol, 0.06 volume of ampholine (pH 3.5–9.5, Pharmacia), and 1% β-OG were cast on electrophoresis film (Sigma). The gel was run under the following conditions: 1,500 V, 5 W, and constant current; the cathode buffer was 1 M NaOH and the anode buffer was 0.05 M sulfuric acid. The purple sbR-1D4 bands were well focused when the voltage reached 900 V (ca. 3 h). The gel was fixed, washed, dried, and stained with Coomassie Brilliant Blue according to the manufacturer's instructions (Pharmacia).

Analytical gel filtration column chromatography for MBP-sbO-1D4 was performed using a Superose 6 column (HR 16/50 column, Pharmacia) on an FPLC (Pharmacia) at room temperature. Buffer 4 was used to prepare the molecular weight standards and the water-soluble fusion protein and Buffer 4 containing 2% SDS was employed to dissociate the aggregated fusion protein; all of the samples were eluted with Buffer 4 at a flow rate of 0.5 mL/min. Gel-filtration chromatography for purified sbR-1D4 and MBP-sbR-1D4 and protein standards was performed with an analytical Superose 12 column (HR 10/30 column, Pharmacia) and Buffer 14 at a flow rate of 0.2 mL/min.

Amino terminal amino acid sequencing

About 100 pm of purified sbO-1D4 were loaded onto a SDS-PAGE gel. After completion of the electrophoresis, the protein was electroblotted onto an Immobilon-pSQ PVDF membrane (Millipore, Bedford, Massachusetts) in Buffer 15 at 6 V for 4 h. The membrane was washed with distilled water for 5 min and stained with 0.25% Coomassie Brilliant Blue R-250 in 40% methanol for 30 min. The membrane was then destained with 50% methanol for 1.5 h and washed with distilled water for 10 min and dried in air. Amino acid sequencing was performed using an Applied Biosystems 477A Protein Sequencer at the University of Kentucky (Matsudaira, 1987).

Quantitation of proteins

Concentrations of MBP-sbO-1D4, sbO-1D4, and sbR-1D4 were determined by the Folin–Lowry method (Robyt & White, 1987) using bovine albumin as a standard. Concentrations of rhodopsin and MBP-sbR-1D4 were determined spectrophotometrically (rhodopsin: ϵ at 500 nm, 40,600 M^{-1} cm^{-1}, Wald & Brown, 1953; bR: ϵ at 555 nm, 50,900 M^{-1} cm^{-1}, Rehorek & Heyn, 1979).

Gene expression

A single colony of DH5α containing pEG-sbOd was grown overnight in 15 mL of LB media containing 50 μg/mL of carbenicillin (LB/Car media) at 37 °C with constant shaking. Subsequently, 10 mL of the overnight culture was transferred into 1 L of fresh LB/Car media containing 0.2% glucose and incubated at 37 °C with vigorous shaking for 3.5 h (OD_{590} = 0.9, mid log phase). IPTG was then added to a final concentration of 0.3 mM and the culture was incubated at 37 °C for an additional 15–17 h. Cells were harvested by centrifugation at 4 °C for 20 min (6,000 rpm, GST rotor, Sorvall).

Preparation of inclusion bodies and purification of MBP-sbO-1D4

The scheme for protein production is shown in Figure 2. The cells were suspended in Buffer 3 (9 mL) supplemented with 1 mM PMSF and 0.2 mg/mL lysozyme and stirred at 4 °C for 20 min. Next, 12 mg of deoxycholic acid was added and the resulting suspension was stirred for an additional 5 min. The suspension was incubated in a water bath at 37 °C with occasional stirring for 10 min or until it became very viscous. DNase I was added (5 μg/mL final concentration), the mixture was incubated at room temperature for 30 min and the inclusion bodies were collected by centrifugation at 4 °C (17,200 rpm for 15 min in a Sorvall SS-34 rotor). The inclusion bodies were washed by resuspending the pellet in 22.5 mL of Buffer 16 containing 1 mM PMSF at 4 °C. The washed inclusion bodies were collected by centrifugation and either dissolved immediately or stored frozen at −20 °C.

Soluble fusion protein was obtained by stirring the washed inclusion bodies in Buffer 16 containing 8 M urea and 1 mM PMSF at room temperature for 2 h. The resulting solution was diluted with an equal volume of Buffer 4 and stirred at room temperature for an additional 1 h. Insoluble materials were removed by centrifugation at 17,200 rpm for 15 min. The supernatant was dialyzed against Buffer 4 (1,000 mL, 3 changes) overnight and centrifuged to obtain the dissolved fusion protein. To purify the fusion protein by amylose affinity chromatography, a sample was loaded onto a column (XK 16/20, Pharmacia; 30 mL of amylose resin, New England Biolabs) equilibrated with Buffer 4 at 4 °C. The column was washed with 10 volumes of Buffer 4 and the desired MBP-sbO-1D4 fusion was eluted with Buffer 4 containing 10 mM maltose.

Factor Xa cleavage of MBP-sbO

Systematic factor Xa cleavage reactions were run using both MBP-sbO purified over the amylose column and protein from the solubilized inclusion bodies (final factor Xa concentrations were typically 20 μg/mL). Reactions were carried out at room temperature in Buffer 4 supplemented with 10 mM maltose, 3 mM

$CaCl_2$, and spanned a range of SDS concentrations (0–0.1%) and times (10 min to 22 h). Reaction mixtures were analyzed by either Coomassie staining or western blotting of 12% SDS-PAGE gels.

Trypsin cleavage of MBP-sbO-1D4

Stock solutions of trypsin (1 mg/mL) were stored in aliquots of 50 μL and 500 μL at −20 °C. The same batch of trypsin was always employed in the pilot and preparative digestions. Favorable digestion conditions were determined by carrying out small-scale reactions at room temperature using an MBP-sbO-1D4 solution in Buffer 4, supplemented with 10 mM maltose, 2 mM $CaCl_2$, and different concentrations of SDS and trypsin for different periods of time. Reactions were stopped by the addition of PMSF to a final concentration of 1 mM and analyzed by SDS-PAGE.

Purification of sbO-1D4

The trypsin reaction mixture from 1 L of culture was reduced to 4 mL, at room temperature, using Centricon-10 (Amicon) concentrators. The resulting solution was passed through a 0.22-μm filter and half of the solution was applied to a Superose 12 column (XK 26/100, Pharmacia). The other half was loaded after all components of the first run were eluted from the column. The sbO-1D4 positive fractions of both runs were pooled and concentrated to 2 mL using a Centricon-10. This sample was loaded onto the same Superose 12 column and eluted with Buffer 17, giving purified sbO-1D4. SDS-PAGE was utilized to determine the fractions containing sbO-1D4.

Reconstitution and purification of reconstituted sbR-1D4

The purified sbO-1D4 was reconstituted in Buffer 18 according to the procedure of Miercke et al. (1991). To do this, a premix composed of 0.2 volumes of 2.5% DMPC and 0.1 volumes of 5% CHAPSO in 0.4 volumes of water was prepared. Next, 0.3 volumes of the gel filtration elute was mixed with 0.7 volume of the premix. This mixture was incubated at room temperature for 10 min and its UV spectrum was taken to estimate the concentration of sbO-1D4. Then all-*trans* retinal was added (fourfold molar excess) and the solution was mixed thoroughly. UV spectral measurements were taken at 2 min and 20 min after the retinal addition. The reconstitution mixture was incubated overnight at room temperature in the dark.

Further purification, under dim light or in the dark, was accomplished as follows. The reconstitution mixture was concentrated to 7 mL with Centricon-10 concentrators and reduced Triton X-100 was added to a final concentration of 0.1%. This solution was dialyzed against Buffer 19 overnight (2 × 1,000 mL). The dialyzed sbR-1D4 solution was loaded onto a Q-Sepharose column (XK 10/16 column, 10 mL of resin, Pharmacia) and the column was eluted with the following FPLC program: flow rate, 0.4 mL/min; 0% Buffer 20 (100% Buffer 21)/0–180 min; 0–30% Buffer 20/181–340 min; 30–100% Buffer 20/341–370 min; 0% Buffer 20/371–380 min. The elute was concentrated to 4 mL, diluted to 24 mL with Buffer 22, and concentrated to 4 mL. This concentration and dilution procedure was repeated twice, after which the solution was diluted to 9 mL with Buffer 22. After filtration through a 0.22-μm membrane filter, the solution was loaded onto a Red A column (XK 10/16 column, Pharmacia; 10 mL of Red A, Amicon). The column was washed with 40 mL of Buffer 22 and sbR-1D4 was eluted with Buffer 23 at a flow rate of 0.5 mL/mL (Miercke et al., 1991).

Small-scale purification of sbR-1D4 from the reconstitution mixture by 1D4-immunoaffinity chromatography was carried out as follows. 1D4-Sepharose resin (0.2 mL) was washed with Buffer 24 (1 mL, 3 times) and then 0.15 mg of sbR-1D4 in 0.5 mL of the reconstitution mixture plus 0.5 mL of Buffer 24 was added and the mixture was mixed on a Nutator at 4 °C overnight. The resin was then thoroughly washed with Buffer 24. The sbR-1D4 was eluted from the resin with the 1′-9′ rhodopsin peptide (50 μg/mL) in Buffer 24 (1 mL, three times).

Regeneration and purification of MBP-sbR-1D4

Partially purified MBP-sbO-1D4 used for trypsin digestion was utilized for reconstitution. Reconstitution was carried out in Buffer 25. The concentration of the fusion protein was 0.35 mg/mL (about 5 μM) and all-*trans* retinal in ethanol was added to a final concentration of 20 μM. The refolding mixture was incubated at room temperature for 2 h and at 4 °C overnight in the dark. The mixture (total volume of 250 mL from 0.5 L of cell culture) was then concentrated to about 70 mL using Centricon-30 concentrators and reduced Triton X-100 was added (0.1% final concentration). The concentrated solution was dialyzed against Buffer 19 (1,000 mL, 2 days), filtered through a 0.45-μm filter, and loaded onto a Q-sepharose column (HiLoad 16/10 Q-sepharose HP, Pharmacia). The elution conditions for MBP-sbR-1D4 were the same as those used for purification of sbR-1D4 except that the flow rate was 1 mL/min. The purple fractions were pooled and concentrated to 20 mL. The resulting solution was diluted to 60 mL with Buffer 22 and then concentrated to 20 mL. This dilution–concentration procedure was performed three times and the final solution was diluted to 40 mL. After filtration through a 0.22-μm filter, the partially purified MBP-sbR-1D4 was loaded onto a Red A column (XK 16/10, Pharmacia; 20 mL of Red A resin, Amicon). The column was washed with 100 mL of Buffer 22 and the pure refolded protein was eluted with Buffer 26 at a flow rate of 1 mL/min.

UV/visible absorption spectra and photocycle measurements of sbR-1D4 and MBP-sbR-1D4

UV/vis absorption spectra were measured on a Lambda 6 UV/VIS spectrometer (Perkin Elmer, Norwalk, Connecticut) using quartz cuvettes. Spectra of dark-adapted sbR-1D4 in Buffer 23 and MBP-sbR-1D4 in Buffer 26 were measured after overnight incubation of the solutions at 4 °C in the dark. Light adaptation was accomplished by exposing the solutions to a 150-W light source for 150 s. For photocycle measurements, sbR-1D4, MBP-sbR-1D4, and bR from purple membrane were purified by chromatography using Red A columns and were dialyzed against Buffer 27 at 4 °C in the dark for 3 days. Photocycle measurements were conducted in the laboratory of Professor Tom Ebrey (University of Illinois).

Acknowledgments

We thank Drs. Koyama, Molday, and Tager for their gifts of monoclonal antibodies, hybridomas, and the 1′-9′ 1D4 peptide, respectively. The Agarwal, Hochstrasser, Haselkorn, Moffat, and Wool laboratories provided many helpful suggestions and allowed us generous use of their

equipment. Purification of the 1D4 monoclonal antibody by Aldona Balciunas, oligonucleotide syntheses and DNA sequencing by the Howard Hughes facility, and photocycle measurements by the Ebrey lab are sincerely appreciated. This work was supported by the Martin D. and Virginia S. Kamen Sustaining Fund for Young Faculty and by the Chicago Community Trust. J.E.G. is a Searle Scholar and a National Science Foundation Young Investigator.

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