PROTEIN EXPRESSION AND PURIFICATION **10,** 61–69 (1997)
ARTICLE NO. PT960704

Functional Expression of Bovine Opsin in the Methylotrophic Yeast *Pichia pastoris*

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Received September 10, 1996, and in revised form November 26, 1996

The methylotrophic yeast *Pichia pastoris* was examined for functional expression of bovine opsin. An expression plasmid was constructed where the bovine opsin gene was placed downstream from the *P. pastoris* alcohol oxidase 1 gene promoter and fused at its amino-terminus to the acid phosphatase secretion signal. Quantitative–competitive PCR analysis of a stable yeast transformant showed that one copy of the opsin gene was integrated into the yeast genome. The expression level in this transformant corresponded to ~0.3 mg of opsin per liter of cell culture (A_{600} = 1.0). Sucrose density sedimentation analysis indicated that the opsin was associated exclusively with the membrane fraction. Similar to retinal opsin, *P. pastoris*-expressed opsin migrated as a single band of ~37 kDa on SDS–PAGE and showed high mannose N-glycosylation. A portion of the expressed opsin (~4–15%) reacted with 11-*cis*-retinal to form the rhodopsin chromophore (λ_{max} 500 nm), and after purification showed ground and excited state spectral characteristics indistinguishable from those of the native pigment. Further, the metarhodopsin-II-mediated G-protein-activating potential of yeast expressed rhodopsin was similar to that of native rhodopsin. These results show that *P. pastoris* cells have the capacity to functionally express bovine opsin.

Rhodopsin is the photoreceptor that mediates dim-light vision. Bovine rhodopsin is composed of the apoprotein opsin, a single polypeptide chain of 348 amino acids, and an 11-*cis*-retinal chromophore (1–4). Opsin contains several posttranslational modifications, including an acetylated amino-terminus, N-glycosylation, a disulfide bridge, and fatty acylation (5–8). The apoprotein folds into a structure of seven transmembrane helices connected by solvent-exposed polypeptide segments on the intradiscal (extracellular) and cytoplasmic surfaces. These seven membrane-spanning helices form a binding pocket for the chromophore (9). Light-induced conformational changes in rhodopsin mediated by retinal isomerization expose binding sites for the heterotrimeric guanine nucleotide-binding protein (G protein)[2], the interface between the receptor and effector molecules in visual transduction (10,11).

An understanding of rhodopsin's tertiary structure and the conformational changes it undergoes upon light activation is essential not only to clarify details of the visual transduction process, but also to gain insight into the severe visual impairments occurring as an immediate consequence of naturally occurring mutations affecting opsin folding and assembly, chromophore formation, and cellular localization (12). However, painstaking attempts to obtain three-dimensional crystals of rhodopsin with reasonable diffraction characteristics have failed in part due to its low stability and inherent light sensitivity (13,14). Thus, a highly efficient expression system capable of producing modified rhodopsin or its defined domains (15,16) in the quantities required for detailed biophysical and structural analyses would be beneficial.

Recombinant rhodopsin has been produced by transient and/or stable transfection of COS-1 (17) and human embryonic kidney 293 cells (18) and by RNA injec-

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[2] Abbreviations used: G protein, guanine nucleotide-binding protein; ROS, rod outer segment; QC-PCR, quantitative–competitive polymerase chain reaction; DM, *n*-dodecyl β-D-maltoside; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; GTPγS, guanosine 5′-[γ-thio]triphosphate.

1046-5928/97 $25.00

tion of *Xenopus laevis* oocytes (19), in baculovirus-infected Sf9 insect cells (20), and by RNA translation in wheat germ extracts (21). Although expressions in COS-1 and 293 cells remain the methods of choice for most structure–function studies on rhodopsin, their use for large-scale production is rather laborious and expensive. High-level production of rhodopsin in baculovirus-infected Sf9 insect cells has recently been improved by regenerating the pigment in the presence of rod outer segment (ROS) lipids and taking advantage of immobilized metal affinity chromatography (22). However, a major disadvantage of this expression system is that it produces a heterogeneous protein population resulting from differential N-glycosylation, and possibly other posttranslational modifications. In this study, we examined whether bovine opsin can be expressed functionally in the methylotrophic yeast *P. pastoris,* an organism capable of producing considerable quantities of foreign proteins (23). The results show that stably transformed *P. pastoris* cells containing a single copy of the opsin gene produce ~0.3 mg of opsin per liter of culture. The yeast-expressed opsin migrates as a single band of ~37 kDa on SDS–PAGE, shows high mannose N-glycosylation, and is membrane-integrated. Upon incubation with 11-*cis*-retinal, a fraction of the membrane-bound opsin forms a chromophore and the resulting pigment exhibits spectral and functional properties similar to those of ROS rhodopsin.

MATERIALS AND METHODS

Materials. The *P. pastoris* expression kit was from Invitrogen.[3] Yeast nitrogen base without amino acids, yeast extract, and proteose peptone were from Difco. Restriction endonucleases and Complete protease inhibitor tablets were from Boehringer Mannheim. Reagents for PCR amplification were from Perkin–Elmer, and Tbr DNA polymerase (PrimeZyme) was from Biometra. Glass beads (0.5 mm) were from Biospec Products (Bartesville, OK), and bovine retinae were from J.A. and W. L. Lawson Co. (Lincoln, NE). The bovine opsin cDNA (bd20) was a gift from Dr. J. Nathans (Johns Hopkins University), and 11-*cis*-retinal was a gift from Dr. R. Crouch (Medical University of South Carolina and the NEI). The K16-155C and rho 1D4 antibodies, which are specific for the amino acid sequences 340–348 and 341–348 of opsin, respectively, have been described (24,25). The sources of other materials used in this investigation have been reported (15).

[3] Certain commercial materials, instruments, and equipment are identified in this report in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment identified are necessarily the best available for the purpose.

Media and buffers. Medium A contained yeast extract (10 g/liter), proteose peptone (20 g/liter), and dextrose (20 g/liter). Medium B contained yeast nitrogen base without amino acids (13.4 g/liter), biotin (0.4 mg/liter), and glycerol (10%, v/v). Medium C contained yeast nitrogen base without amino acids (13.4 g/liter), biotin (0.4 mg/liter), and methanol (0.5%, v/v). Buffer A was 7 mM NaH_2PO_4, pH 6.5, 7 mM EDTA, 7 mM dithioerythritol. Buffer B was 20 mM NaH_2PO_4, pH 6.5, 140 mM NaCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$. Buffer C was the same as buffer B except that it contained 10 g/liter dodecyl maltoside (DM). Buffer D was 20 mM NaH_2PO_4, pH 6.5, 140 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 1 g/liter DM. Buffers A–D were supplemented with protease inhibitor tablets and 1 mM phenylmethylsulfonyl fluoride. Buffer E was 2 mM NaH_2PO_4, pH 6.2, 1 g/liter DM. Buffer F was the same as buffer E except that it contained 35 μM c′-TETSQVAPA peptide, which corresponds to the carboxyl-terminal nine amino acids of bovine opsin.

Cloning of the bovine opsin gene into the expression vector pHIL-S1. The pHIL-S1 plasmid contains a unique *Eco*RI restriction site for cloning downstream from the *P. pastoris* alcohol oxidase 1 (*AOX1*) gene promoter and the acid phosphatase (*PHO1*) secretion signal (S) (Fig. 1). Cloning the opsin gene into the *Eco*RI site results in an in-frame insertion with the secretion signal open reading frame. Therefore, *Eco*RI sites were created at the 5′ and 3′ ends of the bovine opsin gene using the polymerase chain reaction (PCR) and primers 1 (5′-GCGAATTCATGAACGGGACCGAGGGCCCAAA) and 2 (5′-GCGAATTCTTAGGCAGGCGCCACTTGGCTGGTCT). The reaction mixture contained 1 ng of bovine opsin cDNA, 15 pmol of each primer, 200 μM dNTPs, and 1 unit of Tbr DNA polymerase in 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% (v/v) Triton X-100. PCR amplifications were typically 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The amplified product was purified by electrophoresis through agarose, digested with *Eco*RI, and subcloned into the *Eco*RI-linearized pHIL-S1 plasmid. Proper insertion and orientation of the opsin gene were confirmed by restriction enzyme analysis. The entire nucleotide sequence of the cloned PCR product was determined using an Applied Biosystems Model 373A automated DNA sequencer and primers 3 (5′-ATGTTCTCTCCAATTTTGTCCTTG), 4 (5′-CGCACACCCCTCAACTACATCCTG), 5 (5′-CCCCACGAGGAGACCAACAATGAG), and 6 (5′-GTCACCACTCTCTGCTGTGGCAAG). Two single-base changes from the GenBank deposited bovine opsin cDNA sequence (Accession Nos. K00502–K00506) were apparent. First, the codon for Gly-101 in the PCR product was GGA instead of GGG. This difference was noted using both primers 3 and 4 in two separate sequencing experiments. Second, the

codon for Leu-119 in the PCR product was CTG instead of TTG. This difference was observed using primer 4 in two separate sequencing runs.

Transformation of P. pastoris cells with the opsin/pHIL-S1 plasmid. The *P. pastoris* strain GS115 (*his4*) was maintained and transformed by electroporation essentially as described in the manual (Version D) of the *Pichia* expression kit. Briefly, GS115 cells were grown overnight at 30°C in medium A to an A_{600} = 1.4–2.0 and then pelleted by centrifugation at 1500*g* for 5 min at 4°C. The cell pellet was resuspended in 500 ml of ice-cold sterile water and centrifuged again. This same procedure was repeated using 200 ml of ice-cold sterile water and then 1 ml of ice-cold sterile 1 M sorbitol. For transformation, the opsin/pHIL-S1 plasmid (1 μg) was linearized with *Sal*I and mixed with 40 μl of cells. Immediately after the pulse, 1 ml of ice-cold 1 M sorbitol was added and aliquots (200 μl) of the transformation mixture were spread on dextrose/agar plates lacking histidine. After 16–18 h at 30°C, colonies were isolated, cultured in medium A, and screened for opsin expression by immunoblot analysis (see below).

Quantitative–Competitive PCR of transformed P. pastoris. For determining opsin gene copy number, genomic DNA was isolated from a saturated yeast culture grown in medium A at 30°C. Briefly, the cells were harvested by centrifugation at 1500*g* for 5 min, and the pellet was washed with 0.5 ml of sterile water and resuspended in 200 μl of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% (v/v) Triton X-100, 1% (w/v) SDS. After 5 min, acid-washed glass beads (~0.3 mg) and 200 μl of phenol:chloroform/isoamyl alcohol (1:1, v/v) were added to the cell lysate. After vigorous mixing, 200 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA was added and the sample was briefly mixed and centrifuged at 8000*g* for 15 min. The aqueous phase was collected, and the DNA was precipitated with ethanol and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The DNA was further purified by CsCl equilibrium centrifugation.

The competitor for both the opsin gene and the *AOX1* promoter in the quantitative–competitive (QC) PCR analysis (26) was constructed from an opsin/pHIL-S1 plasmid in which the *HIS4* gene was removed by *Bam*HI and *Nde*I digestion (Fig. 1). For the opsin competitor, this plasmid was digested with *Sph*I and *Acc*I to remove 350 bp between positions 549 and 899 of the opsin gene (Fig. 1). The plasmid was then blunt-ended with mung bean nuclease and self-ligated. The *AOX1* competitor was created using primers 7 (5′-GCACGCGTACGGTCTGCTGCTAGTGTAT) and 8 (5′-GCACGCGTTAGGCTACTAACACCATGAC), which were designed against the *AOX1* promoter at positions 101–120 and 231–250, respectively. These primers, which contain sequences for an *Mlu*I restriction site, were used to amplify all the way around the plasmid (27). Following amplification, the PCR product was digested with *Mlu*I and self-ligated, thus creating a plasmid with a 110-bp deletion in the *AOX1* promoter. A dilution series of the plasmid competitor (0.5–20 amol) was added to a PCR mixture containing ~3 ng of genomic DNA and the reactions were performed as described above. For the opsin gene, primers 1 and 2 were used to amplify the entire opsin sequence (1047 bp). For the *AOX1* promoter, primers 9 (5′-TCCAAAGACGAAAGGTTGAA) and 10 (5′-TTGATCTTCTCAAGTTGTCG) were used to amplify a 913-bp portion of this region. Following amplification, the reaction products were separated by electrophoresis through agarose and visualized with ethidium bromide.

Expression of bovine opsin in P. pastoris. The transformed GS115 cells were grown in 25 ml of medium B at 30°C to an A_{600} = 10.0. The cells were harvested by centrifugation at 1500*g* for 5 min and resuspended in 250 ml of medium C in a 1-liter capacity shake flask. After further growth for 2–6 days at 30°C, the cells were harvested and stored at −80°C until use. Several independent clones were characterized by immunoblot analysis using serially diluted K16-155C antibody to select for cells with the highest expression levels. One of these clones, YopspHIL-S1-8, was selected for further characterization.

Preparation of crude membranes from P. pastoris cells. Yeast cultures grown to an A_{600} = 1.5–1.6 were centrifuged at 1500*g* for 5 min, and the pellet was washed once with ice-cold water. The pellet was suspended in buffer A, immediately centrifuged, and resuspended in 2 pellet vol of the same buffer. Four pellet volumes of ice-cold acid-washed glass beads was added and the cells were disrupted by vigorous mixing for 10 min at 4°C. The cell debris was removed by centrifugation at 1500*g* for 5 min and the cell lysate layered on a sucrose cushion (50%, w/v in buffer A) and centrifuged at 120,000*g* for 1 h in a swinging bucket rotor. The interface fraction, which is composed of cellular membranes, was collected and immediately resuspended in buffer B.

Chromophore formation and purification of P. pastoris-expressed rhodopsin. All subsequent manipulations were carried out under dim red light. The crude membrane fraction was incubated with 11-*cis*-retinal (2.5 μM in ethanol) for 2 h at 4°C with gentle agitation. The membranes were collected by centrifugation at 100,000*g* for 30 min and the pellet was solubilized in buffer C for 1 h at 4°C. The insoluble material was removed by centrifugation at 100,000*g* for 1 h. The supernatant, containing the solubilized rhodopsin, was purified by immunoaffinity chromatography essentially as described (17) with some minor modifications. To 15 ml of soluble membrane extract was

added 0.2 ml of immobilized rho-1D4 (binding capacity = 0.3–0.5 μg rhodopsin/μl resin). The mixture was incubated for 3 h at 4°C with gentle agitation, and washed five times with 20 bed vol of buffer D, and then an additional five times with 20 bed vol of buffer E. The bound proteins were eluted from the immobilized antibody with buffer F.

Electrophoresis and immunoblotting. Protein samples were analyzed by SDS–PAGE (28) with a 5% stacking and a 10 or 16% resolving gel. The proteins were visualized by staining with Coomassie blue or transferred to poly(vinyldifluoride) membranes for immunoblot analysis (29). Immunoreactive protein was detected with the rho 1D4 or K16-155C antibodies followed by horseradish peroxidase-conjugated goat anti-mouse IgG. The protein bands were visualized by chemiluminescence.

Other methods. ROS were prepared under dim red light from frozen bovine retinae (30). ROS rhodopsin was solubilized in DM and purified by immunoaffinity chromatography on 1D4-Sepharose as described above. Spectral characterization of rhodopsin and G protein activation assays were performed as described previously (15). Cleavage of oligosaccharide chains with endoglycosidase H and PNGase F and ELISA assays were also performed as described previously (16).

RESULTS

Choice of expression vector. The vector pHIL-S1 (Fig. 1) was chosen for expression of the bovine opsin gene since it contains sequences which code for the *PHO1* signal sequence (S), a native *P. pastoris* secretion signal (31). The rationale behind this choice was that the presence of the secretion signal would likely target the opsin to the plasma membrane. Cloning of the opsin gene into the unique *Eco*RI site results in an in-frame insertion with the secretion signal open reading frame. Further, this insertion point is immediately downstream from the secretion signal cleavage site (31), such that the resulting opsin would contain at most three additional amino acids at its amino-terminus after proteolytic processing. Notably, using a nearly identical vector which lacked the secretion signal (pHIL-D2) failed to produce detectable amounts of membrane-bound opsin (data not shown).

Integration of the bovine opsin gene. Initially, several yeast transformants were screened for opsin expression by immunoblot analysis. Although a number of transformants gave a positive signal, one of these, YopspHIL-S1-8, showed the highest levels of expression. Therefore, this particular transformant was used in all subsequent experiments. Because the opsin/pHIL-S1 plasmid was linearized in the *HIS4* gene (Fig. 1), it was possible to have multiple integration events into the yeast genome. The number of integrants in

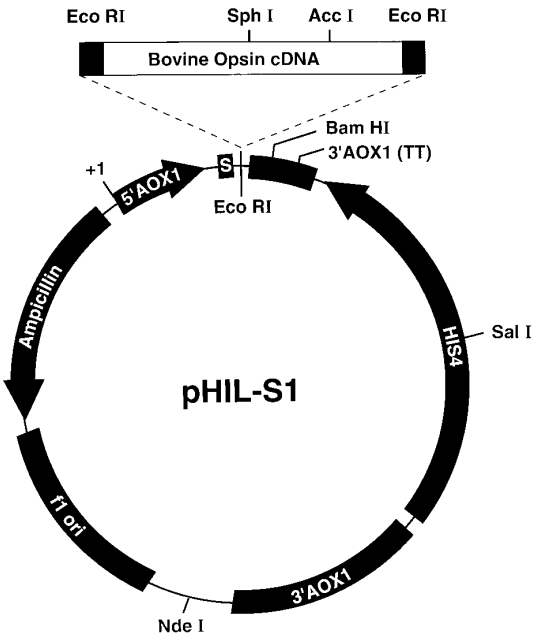

FIG. 1. A schematic diagram showing the main features of the *P. pastoris* expression vector pHIL-S1 and the strategy used for insertion of the bovine opsin cDNA. 5′ *AOX1,* the *P. pastoris* alcohol oxidase 1 gene promoter; S, the secretion signal of the *P. pastoris* acid phosphatase 1 gene which encodes the sequence MFSPILSLEIIL-ALATLQSVFA; 3′ *AOX1* (TT), the native transcription termination and polyadenylation signal from the alcohol oxidase 1 gene; *HIS4,* the *P. pastoris* gene coding for histidinol dehydrogenase; 3′ *AOX1,* sequences from the *AOX1* gene that are 3′ to the transcription terminator; f1 ori, the bacteriophage f1 origin of replication; Ampicillin, the ampicillin resistance gene. For cloning of the bovine opsin gene into the unique *Eco*RI site of the vector, *Eco*RI sites were created at the 5′ and 3′ ends of the opsin gene using PCR (see Materials and Methods for details). Prior to transformation of GS115 cells, the plasmid was linearized at the unique *Sal*I site.

YopspHIL-S1-8 was analyzed using QC PCR to determine the relative ratio of the *AOX1* promoter copy number to the opsin gene copy number. If there was only a single integration event, then there would be two copies of the *AOX1* promoter (one chromosomal plus one from the opsin/pHIL-S1 vector) to one copy of the opsin gene. If there were multiple integration events, then the ratio should be less than 2:1 (e.g., 3:2 or 4:3), depending on the number of copies. Amplification of the *AOX1* competitor most closely equaled that of the *AOX1* target at 5 amol of competitor (Fig. 2A), while amplification of the opsin competitor equaled that of the opsin target at approximately 2.5 amol of competitor (Fig. 2B). This is consistent with a 2:1 ratio (*AOX1*:opsin), suggesting that a single copy of the opsin gene was integrated into the YopspHIL-S1-8 genome.

Expression of the bovine opsin gene. To examine for stable opsin expression, extracts were prepared from YopspHIL-S1-8 cells at 48 h postinduction and analyzed by immunoblotting following SDS–PAGE. No im-

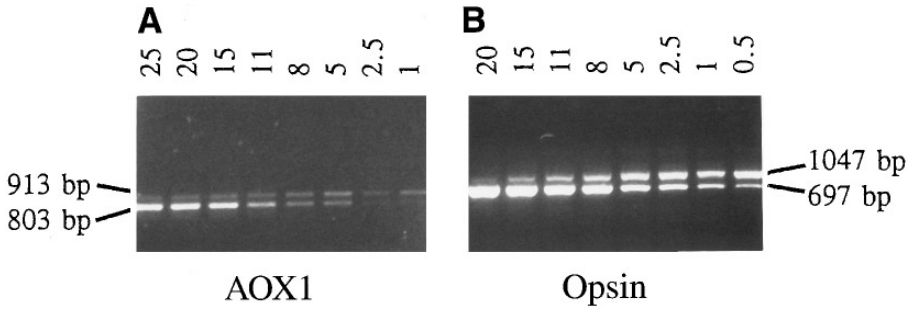

FIG. 2. Quantitative–competitive PCR analysis for determining bovine opsin gene copy number in transformed *P. pastoris*. The relative ratio of the (A) *AOX1* promoter to the (B) opsin gene in YopspHIL-S1-8 was estimated by amplifying genomic DNA in the presence of a dilution series of the *AOX1* or opsin plasmid competitors (see Materials and Methods for details). The numbers above each lane indicate the amount of competitor (in amol) added to each PCR mixture. The reaction products in (A) correspond to the *AOX1* promoter fragment (913 bp) and the *AOX1* promoter fragment with a 110-bp deletion (803 bp). The reaction products in (B) correspond to the entire opsin gene (1047 bp) and the opsin gene with a 450-bp deletion (697 bp).

munoreactive material was detected in extracts from uninduced cells (Fig. 3A, lane 1). Only those cells induced with methanol gave an immunoreactive band which migrated at ~37 kDa (Fig. 3A, lane 2). Thus, the *P. pastoris*-expressed opsin migrates slightly faster on SDS–PAGE than ROS opsin (Fig. 3A, lane 3). The levels of opsin expression were estimated by comparing both immunoblots and ELISA assays on cell extracts with known amounts of ROS rhodopsin. Taken together, the results show that 1 liter of induced cells (A_{600} = 1.0) produces about 0.27 ± 0.03 mg of opsin. Since a liter of yeast grown to A_{600} = 1.0 yields approximately 40 mg of membrane protein (32), the expression level of opsin accounts for roughly 0.6–0.75% of the total membrane protein.

To determine whether the opsin was membrane-integrated, whole-cell extracts were layered on a sucrose cushion and centrifuged. Three different fractions were apparent and each was collected and analyzed for opsin content by immunoblotting. Virtually all of the opsin was found in the membrane-containing interface fraction located on the border of the buffer/sucrose cushion (Fig. 3B, lane 1). Only a negligible amount of opsin was observed in the slightly turbid sucrose solution, while none was detected in the heavy pellet of debris (Fig. 3B, lanes 2 and 3, respectively). Although this analysis does not show the specific location of the opsin in the cells, the above results indicate that it is integrated into the membrane.

The presence and extent of N-glycosylation on *P. pas-*

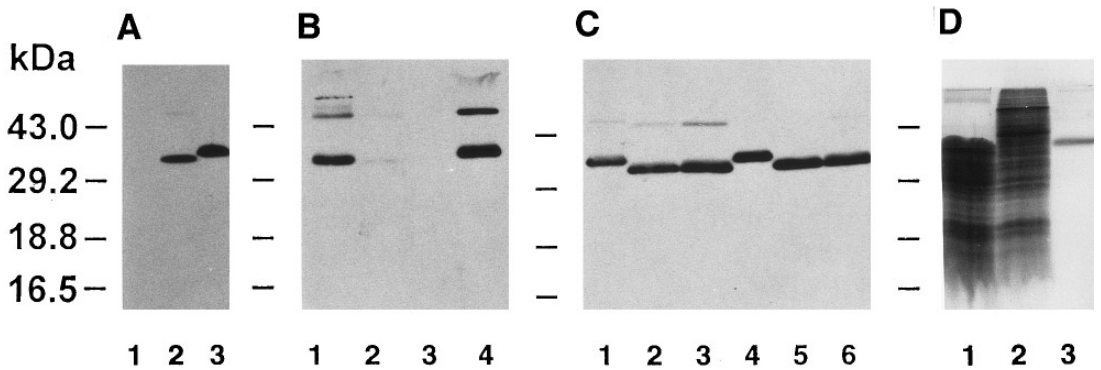

FIG. 3. Expression of bovine opsin in *P. pastoris*. (A) Whole-cell extracts from ~5 × 10^5 cells before (lane 1) and after (lane 2) induction with methanol (medium C). Lane 3 contains 10 ng of ROS rhodopsin. (B) Fractions obtained from whole-cell extracts after sucrose density sedimentation analysis. Lane 1, membrane containing interface fraction; lane 2, 50% sucrose phase; lane 3, pellet of debris; lane 4, 20 ng of ROS rhodopsin. (C) Effect of glycosidase treatment on yeast-expressed and ROS rhodopsins. Lanes 1 and 4, yeast-expressed and ROS rhodopsins, respectively; lanes 2 and 5, endoglycosidase H-treated yeast-expressed and ROS rhodopsins, respectively; lanes 3 and 6, PNGase-F-treated yeast-expressed and ROS rhodopsins, respectively. (D) Purification of yeast-expressed rhodopsin. Lane 1, whole-cell extracts from ~5 × 10^5 cells; lane 2, crude membrane preparation from ~5 × 10^5 cells; lane 3, immunoaffinity-purified yeast-expressed rhodopsin (1 μg). The samples were analyzed by reducing SDS–PAGE and the proteins visualized by immunoblotting with the rho 1D4 antibody (A–C) or Coomassie blue staining (D). Positions of molecular size standards are shown at the left in kilodaltons. The immunoreactive bands which migrate above the the 43-kDa standard (B and C) represent opsin dimers.

toris-expressed opsin was compared with that of ROS opsin by glycosidase treatment. The yeast-expressed apoprotein showed a faster migrating polypeptide after treatment with endoglycosidase H and PNGase F (Fig. 3C, lanes 2 and 3). Thus, like ROS opsin (Fig. 3C, lanes 4–6), yeast-expressed opsin is high-mannose N-glycosylated.

Purification and characterization of the expressed rhodopsin. Membranes from a culture of YopspHIL-S1-8 were incubated with 11-*cis*-retinal, solubilized with DM, and purified on immobilized rho-1D4 antibody. Figure 3D shows some of the products obtained during the different steps of rhodopsin purification. While numerous proteins were present in the whole-cell extract and membrane preparation (Fig. 3D, lanes 1 and 2), only a single polypeptide of ~37 kDa was present in the antibody column eluate (Fig. 3D, lane 3).

Ultraviolet–visible spectroscopy of the purified yeast-expressed pigment showed the characteristic rhodopsin spectrum with absorption maxima at 280 and 500 nm (Fig. 4A). The spectrum shown was obtained from a sample which was eluted from the immobilized rho 1D4 antibody under conditions of low pH and low ionic strength (buffer F). Here, the ratio of the absorbance at 280 to that at 500 nm was 1.6–1.7, which is characteristic of purified rhodopsin (33). However, preparations of *P. pastoris* rhodopsin which were eluted from the immunoaffinity matrix with high ionic strength buffers showed considerably higher A_{280}/A_{500} ratios (~3–5). This increase is presumably due to the coelution of both correctly folded regenerated rhodopsin and misfolded opsin that does not bind retinal (34). Assuming an extinction coefficient of 40,600 $M^{-1} \cdot cm^{-1}$ for the rhodopsin chromophore (35), the yield of correctly folded rhodopsin after purification varied from ~10 to 40 μg for a 1-liter culture of A_{600} = 1.0. The yeast-expressed rhodopsin also showed a shift in λ_{max} to 380 nm upon illumination and formed the protonated retinyl-Schiff base (λ_{max} 440 nm) upon acidification.

To test if the yeast-expressed rhodopsin activated G protein, *in vitro* GTPγS binding assays were performed. The yeast-expressed rhodopsin catalyzed light-dependent GTPγS binding by G protein to about 90% of the level of ROS rhodopsin (Fig. 4B).

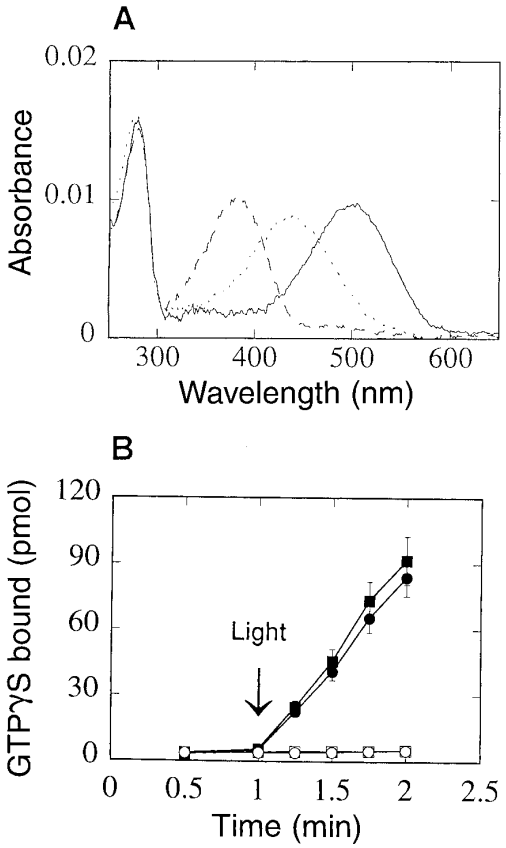

FIG. 4. Spectral and functional characterization of *P. pastoris*-expressed rhodopsin. (A) Ultraviolet–visible spectra of yeast-expressed rhodopsin. Crude membrane preparations from cells expressing opsin were incubated with 11-*cis*-retinal and solubilized in DM, and the protein was purified by immunoaffinity chromatography on 1D4-Sepharose. Spectra were recorded in the dark (—), after illumination (>495 nm) for 10 s (– –), and after acidification to pH ~1.9 (---). (B) Light-dependent activation of G protein by yeast-expressed and ROS rhodopsins. Time course for the reaction catalyzed by ROS (circles) and yeast expressed (squares) rhodopsins. The assay mixture contained 2 nM rhodopsin, 2 μM G protein, and 4 μM [^{35}S]GTPγS. Closed symbols are data for the light reaction and open symbols are data for the dark reaction. The results shown are averages ± standard error from two independent determinations.

DISCUSSION

Heterologous expression of membrane proteins in yeast has long been the focus of attention of many research groups (for a review, see Ref. 36). A partial list of seven-transmembrane-helix receptors that have been functionally expressed in various species of yeast includes the β_2-adrenergic receptor, bacterioopsin, the m1 and m5 muscarinic acetylcholine receptors, the D_{2S} dopamine receptor, and the 5-HT_{5A} serotonin receptor (37–42). In this paper, the methylotrophic yeast *P. pastoris* was tested for heterologous expression of bovine opsin. Several key features of the *P. pastoris* expression system made it an attractive candidate for heterologous expression of opsin. First, the gene of interest is placed under the transcriptional control of the tightly regulated and highly inducible *AOX1* promoter. In some cases, heterologous expression of foreign genes from this promoter has yielded gram quantities of the desired recombinant protein per liter of yeast culture (43). Second, the gene of interest can also be fused at its amino-terminus to a native *P. pastoris* secretion signal (e.g., PHO1), a feature which may influence the membrane localization of the expressed protein. Fi-

nally, *P. pastoris* cells possess the machinery necessary for many posttranslational modifications found in higher eukaryotes, including N-glycosylation, disulfide formation, and fatty acylation.

Several lines of evidence suggest that *P. pastoris*-expressed opsin is similar to ROS opsin. First, the yeast-expressed apoprotein shows a single band of ~37 kDa on SDS–PAGE (Fig. 3), an apparent molecular mass that is slightly lower than that of ROS opsin. Although the amino acid composition of the expressed opsin was not determined experimentally, it most likely contains an additional Arg, Glu, and Phe at its amino-terminus after secretion signal processing. This likelihood is based on observations by Weiss *et al.* (42) who showed that expression of a 5-HT_{5A} serotonin receptor/pHIL-S1plasmid in *P. pastoris* resulted in cleavage of the 20-amino-acid *PHO1* secretion signal. Second, based on glycosidase sensitivity, the yeast-expressed opsin, like ROS opsin, appears to be high-mannose N-glycosylated (Fig. 3C). Although the precise nature of the oligosaccharide moieties on the *P. pastoris* opsin is not evident from this work, it is possible that differences in carbohydrate composition between this and ROS opsin may contribute to the slight difference in apparent molecular mass. Opsin expressed in COS-1 and 293 cells is heterogeneously N-glycosylated, as evidenced by its tendency to smear on SDS–PAGE (17,18). On the other hand, opsin preparations from baculovirus-infected Sf9 cells show additional immunoreactive bands which have been attributed to the nonglycosylated form of the protein (20,22). Importantly, no heterogeneously glycosylated material or additional immunoreactive bands were apparent in preparations of the yeast-expressed opsin. Third, and perhaps most important, a portion of the *P. pastoris*-expressed opsin binds exogenously added 11-*cis*-retinal to form the characteristic rhodopsin chromophore (Fig. 4A). The resulting pigment was immunopurified to apparent homogeneity (Fig. 3D) and undergoes the same light-dependent and acid-induced conformational changes as ROS rhodopsin. Finally, the yeast-expressed rhodopsin activates G-protein in a light-dependent manner (Fig. 4B). Although the reason for the slightly reduced signaling capacity is not clear at the present time, it may also be related to differences in carbohydrate composition since N-glycosylation of bovine opsin appears to be important for the G-protein-activating potential of metarhodopsin II (44).

The yield of *P. pastoris*-expressed rhodopsin after purification varied from ~10 to 40 μg per liter of shake flask culture (~5×10^{10} cells), or roughly 4–15% of the total opsin apoprotein produced. For comparison, an equivalent number of transiently transfected COS-1 cells would produce ~2 mg of rhodopsin (17), whereas the same number of baculovirus-infected Sf9 cells would yield 3–4 mg of rhodopsin (22). Although *P. pastoris* cells can be grown and induced for longer periods of time to produce higher milligram amounts of opsin, this does not dramatically change the regenerability of the opsin. These results suggest that a single copy of the opsin gene is sufficient for high-level expression and that other factors influence the ability of the opsin to adopt a retinal-binding conformation. Lack of N-glycosylation was reported earlier to reflect impaired opsin folding and/or assembly, as judged by loss of regeneration capacity (45). However, this does not seem to be relevant to the present expression system since no additional bands which could be attributed to nonglycosylated or hyperglycosylated opsins were observed on the immunoblots. One possible explanation for the lack of quantitative regeneration might be that *P. pastoris* membranes do not contain adequate or appropriate lipids which provide more favorable conditions for the spontaneous regeneration of rhodopsin. This lack of lipids could result in a decreased rate or extent of regeneration or affect the stability of regenerated rhodopsin during purification in detergent solution. In this context, a large excess of ROS lipids was added to Sf9 insect cell lysates containing baculovirus-expressed opsin to favor the stability and regeneration level of rhodopsin (22). Our preliminary results show that addition of exogenous retinal lipids is also beneficial for the regeneration of *P. pastoris*-expressed opsin (N. G. Abdulaev and K. D. Ridge, unpublished observations).

Although unsuccessful attempts at functional expression of bovine opsin in *Saccharomyces cerevisiae* have been reported (17,21), Khorana and colleagues recently succeeded in this species of yeast (46). Their preparation of yeast-expressed opsin also partially formed a chromophore with 11-*cis*-retinal and showed spectral and functional properties similar to native rhodopsin. Identifying additional parameters which lead to increased levels of chromophore formation for opsin expressed in both species of yeast is likely to be mutually beneficial. Such success would allow for the high-level production of mutants and metabolically labeled analogs of opsin, providing an extremely powerful tool for more extensive and detailed structure–function studies.

ACKNOWLEDGMENTS

We thank Dr. Prasad Reddy for DNA sequencing, Dr. James M. Cregg for technical advice, Stephen S. J. Lee for technical assistance, and Drs. Paul A. Hargrave and H. Gobind Khorana for discussions. This work was supported by the National Institute of Standards and Technology and by Grants EY11112 (to K.D.R.) and EY06641 (to M.P.P.) from the National Eye Institute.

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