

Structure and function in rhodopsin: High level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines

(G-protein coupled receptor/11-*cis*-retinal/glycosylation/metarhodopsin II/immunoaffinity chromatography)

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ABSTRACT Stable mammalian cell lines harboring a synthetic bovine opsin gene have been derived from the suspension-adapted HEK293 cell line. The opsin gene is under the control of the immediate-early cytomegalovirus promoter/enhancer in an expression vector that also contains a selectable marker (*Neo*) governed by a relatively weak promoter. The cell lines expressing the opsin gene at high levels are selected by growth in the presence of high concentrations of the antibiotic geneticin. Under the conditions used for cell growth in suspension, opsin is produced at saturated culture levels of more than 2 mg/liter. After reconstitution with 11-*cis*-retinal, rhodopsin is purified to homogeneity in a single step by immunoaffinity column chromatography. Rhodopsin thus prepared (>90% recovery at concentrations of up to 15 μ M) is indistinguishable from rhodopsin purified from bovine rod outer segments by the following criteria: (i) UV/Vis absorption spectra in the dark and after photobleaching and the rate of metarhodopsin II decay, (ii) initial rates of transducin activation, and (iii) the rate of phosphorylation by rhodopsin kinase. Although mammalian cell opsin migrates slower than rod outer segment opsin on SDS/polyacrylamide gels, presumably due to a different *N*-glycosylation pattern, their mobilities after deglycosylation are identical. This method has enabled the preparation of several site-specific mutants of bovine opsin in comparable amounts.

Wild-type bovine rhodopsin is readily available in relatively large amounts. However, structure–function studies require mutants that necessitate expression of the corresponding opsin genes for their preparation. With the aim of preparing such mutants in amounts larger than those currently available by transient transfection of the mammalian COS-1 cell line, alternative expression systems are being explored. In an accompanying paper, the yeast *Saccharomyces cerevisiae* was investigated for expression of a synthetic opsin gene (1). We now report on the development and use of stable mammalian cell lines for the expression of a synthetic opsin gene and several of its mutants. Stable mammalian cell lines, particularly those derived from HEK293 cells, have been used frequently for expression of a variety of eukaryotic water-soluble and integral-membrane proteins (2–5). The expression of bovine opsin and several of its mutants in these cells has previously been described by Nathans and coworkers (6–8). In this paper, stable mammalian cell lines containing a synthetic bovine opsin gene have been derived from a suspension-adapted HEK293 (293S) cell line. The use of a vector (3) designed to target the opsin gene integration into chromosomal regions that favor high level expression has enabled the production of opsin in milligram amounts per liter of the cell cultures grown to saturation. Following reconstitution of the opsin with

11-*cis*-retinal, rhodopsin has been purified to homogeneity in a single step by immunoaffinity chromatography. By a variety of chemical and functional criteria, 293S rhodopsin has been demonstrated to be indistinguishable from rhodopsin prepared from bovine rod outer segments (ROS). This method has enabled the preparation of a number of opsin mutants in satisfactory amounts.‡

MATERIALS AND METHODS

Materials. Frozen bovine retinae were from the J. A. Lawson Co. (Lincoln, NE). Dodecyl- β -1-maltoside (DM) was from Anatrache (Maumee, OH). Protein A-Sepharose was from Repligen. CNBr-activated Sepharose 4B was from Pharmacia. All [γ -³⁵S]GTP and [γ -³²P]ATP were from DuPont/NEN. *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (Ultrasol grade) was from Calbiochem. PNGase-F was from New England Biolabs. Nitrocellulose filters (BA-85) were from Schleicher & Schuell. Mini columns (12 mm inside diameter \times 107 mm) for chromatography were from Pierce. The enhanced chemiluminescence (ECL) detection kit was from Amersham. DNA purification columns were from Qiagen (Chatsworth, CA). The Sequenase DNA sequencing kit was from United States Biochemical. Restriction enzymes were from Boehringer Mannheim and New England Biolabs.

293S cells, a suspension-adapted variant of HEK293 (ATCC no. CRL 1573) were provided by J. Nathans (Johns Hopkins School of Medicine). Plasmid pACHenc was donated by A. Schafferman (Israel Institute for Biological Research). Geneticin (G418) was from GIBCO/BRL. DMEM (Dulbecco's modified Eagle's medium), DMEM/F12, DMEM/Ham's nutrient mixture F12 (1:1), trypsin, EDTA, penicillin, streptomycin, and L-glutamine were purchased from GIBCO/BRL or Irvine Scientific. HB-Gro was from Irvine Scientific. Fetal bovine serum (FBS) was from Sigma.

The nonapeptide corresponding to the rhodopsin carboxyl-terminal 9 amino acids was synthesized at the Biopolymer Laboratories, Cancer Center Research Institute of the Massachusetts Institute of Technology.

Buffers used were as follows: *Buffer A* (137 mM NaCl/2.7 mM KCl/1.5 mM KH₂PO₄/8 mM Na₂HPO₄, pH 7.2). *Buffer B* (Buffer A with 1% DM). *Buffer C* {10 mM 1,3-bis[tris(hydroxymethyl)-methylamino] propane, pH 6.0 with 0.1% DM}. *Buffer D* (Buffer C + 100 μ M nonapeptide). *Buffer E* (Buffer A + 0.1% DM + 100 μ M nonapeptide). *Buffer F* {(2 \times) 50 mM/[*N,N*]-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid]/250 mM NaCl/1.5 mM Na₂HPO₄, pH 7.02}.

Abbreviation: ROS, rod outer segment(s).

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Where indicated, buffers contained 1 mM each of the protease inhibitors, phenylmethylsulfonyl fluoride, benzamide, aprotinin, pepstatin A, and leupeptin.

Methods. The preparation of ROS from frozen bovine retinae was as described (9). Rhodopsin was purified by immunoaffinity chromatography from solubilized ROS membranes or from COS-1 solubilized cell extracts using rho 1D4-Sepharose beads as described (10), except that the protein was eluted with the C-terminal nonapeptide used at 100 μ M concentration. Transducin was purified from bleached ROS as described by Fung (11). The rates of its activation were determined at 19–21°C by measuring the $G_{T(\alpha)}:[\gamma\text{-}^{35}\text{S}]\text{GTP}$ complex using the filter binding assay as described (12). Rhodopsin kinase was purified from bleached ROS as described by Palczewski (13). Initial rates of rhodopsin phosphorylation were determined using a filter binding assay (R.L.T., C. Creuzenet, P.J.R., and H.G.K., unpublished). The reaction mixture contained 0.1 μ M rhodopsin, 1.15 μ g rhodopsin kinase, 0.02% DM, 100 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (≈ 2700 cpm/pmol), and 2 mM MgCl_2 in 1050 μ l of 20 mM 1,3-bis[tris(hydroxymethyl)-methylamino] propane, pH 7.5. The reaction was initiated by illumination from a slide projector lamp, and samples (200 μ l) were removed at 1-min intervals.

UV/Vis Absorption Spectroscopy. This was carried out using a Lambda 7 spectrophotometer (Perkin-Elmer). The molar extinction coefficient used for rhodopsin was 42,700 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 500 nm. For spectra after photobleaching, samples were illuminated for 20 sec. using light (λ , >495 nm) from a fiber optic light source. The decay of metarhodopsin II was monitored by fluorescence increase as described (14). Concentrations of reconstituted rhodopsin in solubilized cell extracts were estimated by difference spectral analysis. For this the UV/Vis absorption spectrum of a solubilized sample was recorded. The spectrum was recorded again immediately after photobleaching for 30 sec. The difference between the two spectra at 500 nm was used to calculate the rhodopsin concentration in the sample.

Protein concentration was determined by the procedure of Bradford (15). For cleavage of the oligosaccharide chains, rhodopsin (0.5 μ g) was incubated in 10 μ l with 25 units of PNGase-F for 3 hr at 37°C. SDS/PAGE was carried out according to Laemmli (16).

Construction of the Expression Plasmid, pACHrhoC. An *EcoRI*–*NotI* DNA fragment containing a synthetic bovine opsin gene was excised from pMT4 (17). Expression vector pACHenc was kindly provided by A. Shafferman (Israel Institute for Biological Research). This vector is a derivative of the vector described by Velan *et al.* (3). pACHenc was digested with *EcoRV* and *Sall*, and the single-stranded ends in both the vector and the fragment were filled using the Klenow fragment of DNA polymerase I and dNTPs. The vector DNA was then treated with calf intestinal phosphatase. Both the vector and the opsin fragment were purified by passage through agarose gels and used in blunt-end ligation reactions. The ligation mixtures were then introduced into *Escherichia coli* (DH1) by the procedure of Inoue *et al.* (18). Plasmid DNA was prepared from ampicillin-resistant colonies and analyzed by digestion with *KpnI*, which cuts asymmetrically in the opsin gene and once in the vector. Recombinant plasmids with the opsin gene in the correct orientation were prepared from *E. coli* cultures using anion exchange columns (Qiagen). The opsin:vector junctions in the final construct (Fig. 1) were confirmed by DNA sequence analysis using opsin gene primers EX16 and PB9 (19).

Construction of Stable Cell Lines Containing the Opsin Gene. 293S cells were maintained in a humidified incubator at 37°C under 5% $\text{CO}_2/95\%$ air in DMEM/F12 supplemented with heat-treated FBS (10%), penicillin G (100 units/ml), streptomycin (100 μ g/ml), and glutamine (292 μ g/ml). 293S cells were transfected using the method of Chen and Okayama

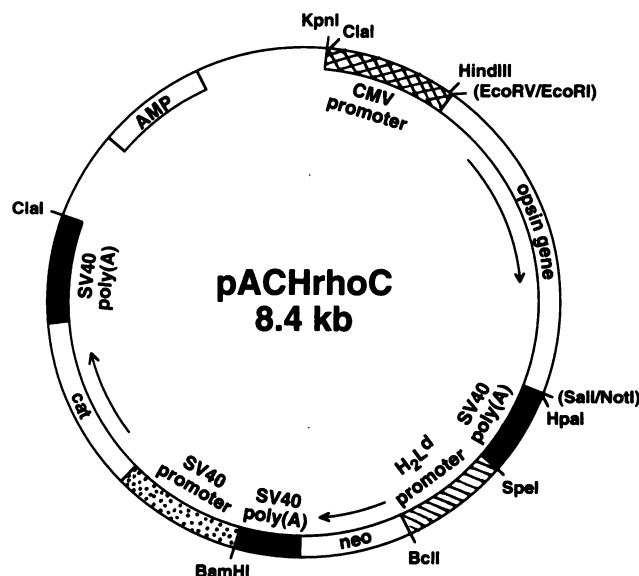


FIG. 1. The vector pACHrhoC used for preparation of stable 293S cell lines harboring the bovine opsin gene. The construction of the vector is described in *Materials and Methods*. The synthetic opsin gene is under control of the cytomegalovirus promoter, whereas the neomycin (neo) resistance gene is under the control of the weak H_2L^d promoter. The restriction sites are indicated. Restriction sites in parentheses, that were present in the starting vector and the opsin gene, were removed by repair to blunt ends for ligation in vector construction.

(20) as modified by O'Mahoney and Adams (21). Briefly, 293S cells in exponential phase were trypsinized and reseeded at a density of $1\text{--}2 \times 10^6$ cells per 10-cm plate in 10 ml DMEM (supplemented as for DMEM/F12). After 25 hr, one cell plate was treated with the above plasmid DNA as follows. The DNA (30 μ g) in 500 μ l of CaCl_2 (0.25 M) was mixed with 500 μ l of buffer F; the mixture was kept for 1 min at room temperature and then added directly to the 293S cell plate. The cells were then transferred to a humidified incubator and incubated under 3% CO_2 at 35°C for 19 hr. The cells were washed once with 10 ml of buffer A, DMEM/F12 (10 ml) was added, and the cells incubated for a further 23 hr under 5% CO_2 at 37°C. They were then trypsinized and replated at about 1×10^6 cells per 10-cm dish and incubated for a further 20 hr. The medium was then replaced with fresh medium containing geneticin and thereafter replaced every 2–3 days until colonies formed 12–15 days later. Colonies were isolated using cloning rings and expanded through 24-well (1 ml), 6-well (3 ml), and finally single 10-cm (10 ml) dishes. Geneticin concentration was maintained throughout these operations. The expression of opsin was then estimated as follows. All procedures were performed on ice or at 4°C. Intact cells were dislodged from a 10-cm dish by washing with 10 ml of buffer A and pelleted by centrifugation. The cells were incubated in the dark in 1 ml of buffer A containing 11-*cis* retinal (5 μ M) and protease inhibitors for 2 hr. The cells were then solubilized in 0.7 ml of buffer B containing protease inhibitors. Cell lines producing opsin were then identified by difference spectrum analysis. For opsin mutants, solubilized whole-cell extracts from 24-well plates were first transferred to nitrocellulose by dot-blot and screened by immunodetection using monoclonal antibody rho 1D4 followed by ECL detection. Opsin-positive cell lines were then expanded, and opsin expression levels were quantified by difference spectrum analysis.

Growth of Cells in Suspension and Reconstitution of Opsin with 11-*cis*-Retinal. Suspension cultures were set up using 1-liter spinner flasks containing 500 ml HB-Gro supplemented

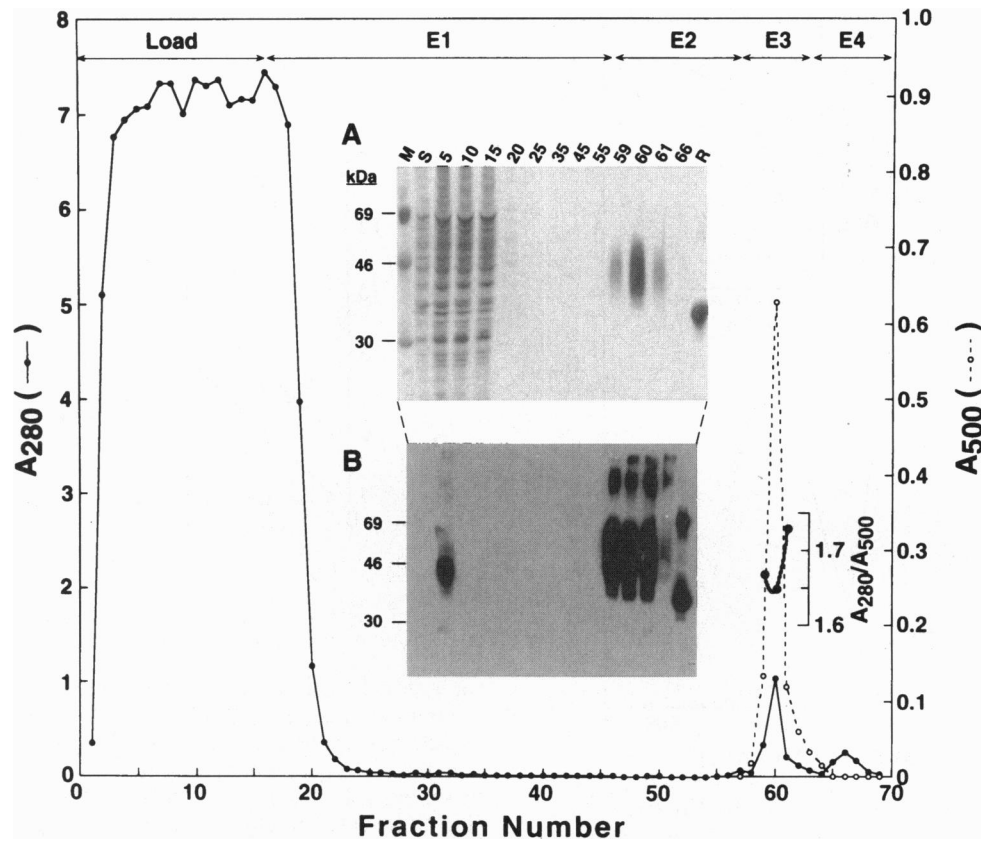


FIG. 2. Purification of rhodopsin reconstituted from opsin produced from an 293S-derived cell line (550 ml of cell suspension culture) on a rho 1D4-Sepharose column. Elution buffers were as follows: E1, buffer B; E2, buffer C; E3, buffer D; E4, buffer E. Absorption (A_{280} and A_{500}) of the fractions eluted were recorded as were A_{280}/A_{500} ratios of the fractions containing rhodopsin. (Inset) SDS/PAGE (10% gel) pattern of selected fractions eluted from column: (A) as visualized by silver stain and (B) immunodetection after electroblotting to nitrocellulose. M, molecular weight standards; S, solubilized cell extract; R, purified ROS rhodopsin.

with FBS (10%), penicillin, and streptomycin. Cell lines were first grown to confluence in two 15-cm culture dishes containing 25 ml of DMEM/F12 with the appropriate concentration of G418. Spinner flasks were inoculated using $4-6 \times 10^7$ cells and incubated for 6-7 days in the absence of geneticin. All further manipulations were performed on ice or at 4°C. Cells from saturated cultures (550 ml) were harvested by centrifugation, washed twice with 50 ml of buffer A, and resuspended in 10 ml buffer A containing protease inhibitors. All further manipulations were performed under dim red light. Cells were incubated with 11-*cis*-retinal (two 25 μ l additions of a 10-mM stock solution) over 3 hr and were then pelleted by centrifugation. Cell pellets were treated with end-over-end mixing for 2 hr in 20 ml of buffer B containing protease inhibitors. The concentration of rhodopsin solubilized in the buffer was estimated by difference spectrum analysis.

Immunoaffinity Purification of 293S Rhodopsin. A column (12 mm \times 107 mm) (5 ml) was packed with 1.6 ml of rho 1D4-Sepharose beads (binding capacity 0.8-1.0 μ g rhodopsin/ μ l settled beads). (Typically, the amount of rho 1D4-Sepharose used was about 10% in excess of the total binding capacity needed for the estimated rhodopsin content in the crude extract.) The column was degassed, equipped with upper and lower porous discs (frits), and pre-equilibrated with buffer B (5 ml) prior to use. Subsequent operations were all in the dark. A solution (about 20 ml) of the DM-solubilized rhodopsin from a 550-ml culture was applied to the column and allowed to flow by gravity (≈ 0.6 ml/min). Flow-through was collected in fractions of about 1.5 ml. The column was then washed (gravity flow) using 50 ml of buffer B followed by 13 ml of buffer C. Rhodopsin was eluted using buffer D at a reduced flow rate of about 0.05 ml per min. Fractions (1.3 ml)

were collected and the eluant was then changed to buffer E for collection of further fractions.

RESULTS

293S Stable Cell Lines Expressing Opsin. The vector pACHrhoC (Fig. 1) was constructed by replacing the human acetylcholinesterase gene in vector pACHenc (3) with the synthetic opsin gene. This vector was determined by restriction analysis to contain the opsin gene downstream from the cytomegalovirus promoter/enhancer and upstream from the simian virus 40 poly(A) sequence. The correctness of the junctions at both 5' and 3' ends of the opsin gene with the vector DNA was confirmed by sequence analysis. This plasmid was used for stable transfection of the 293S cell line, and selection was with geneticin at concentrations of 0.8, 1.0, or 2.0 mg/ml. Resulting colonies were expanded to 10-cm culture dishes, and the total cells thus obtained were tested for opsin content. This was done by the standard treatment with 11-*cis*-retinal, solubilization in buffer B, and analysis by the difference spectrum analysis of the absorption spectra in light and in dark. Typically, 40-50% of the G418^r cell lines were positive for opsin production but showed a range of expression levels. The maximum expression level obtained using 0.8 mg/ml of G418 selection was 9 μ g of rhodopsin per 10-cm culture dish (about 1×10^7 cells). By increasing the selection level to 2.0 mg/ml, a cell line producing wild-type rhodopsin at about 20 μ g per 10-cm culture dish was obtained. For mutants, especially those that express to lower levels in COS-1 cells, more colonies were screened (20-50) and the geneticin concentration was raised to 3 mg/ml. In addition, opsin-positive cell lines were first

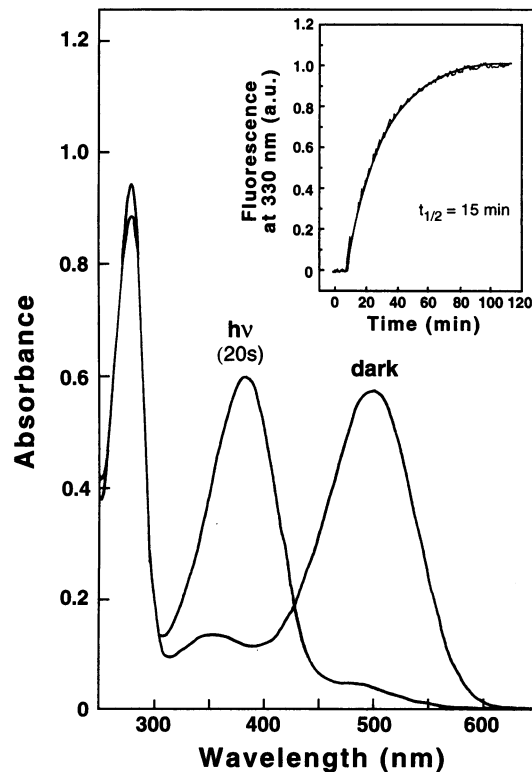


FIG. 3. UV/Vis absorption spectra of purified rhodopsin from 293S cells. The spectra shown are those in the dark (A_{280}/A_{500} ratio 1.65) and after illumination at $h\nu > 495$ nm for 10 sec. (Inset) Rate of metarhodopsin II decay as followed by fluorescence increase at 330 nm.

identified by dot-blot analysis before quantitation by difference spectrum analysis.

293S Cell Growth in Suspension and Rhodopsin Purification. The conditions for cell growth in suspension (2) were modified by using the richer HB-Gro (Irvine Scientific) medium supplemented with 10% FBS. A 550-ml culture of the cell line was harvested, treated with 11-*cis*-retinal, and solubilized

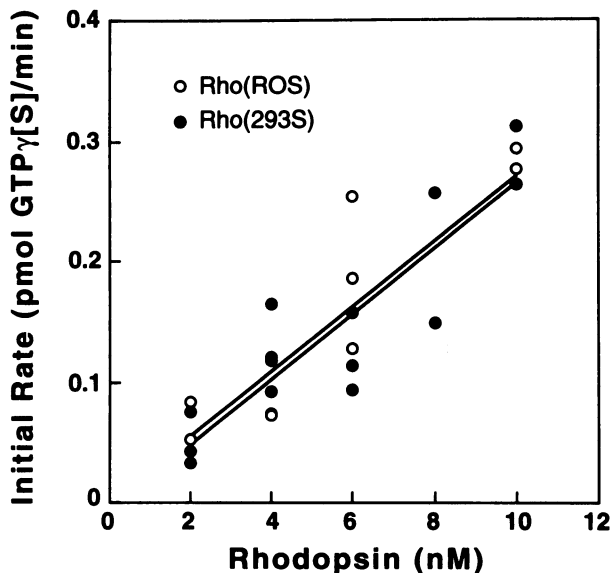


FIG. 4. Initial rates of transducin activation as measured by $G_T(\alpha):[\gamma\text{-}^{35}\text{S}]\text{GTP}$ complex formation. Shown are the rates obtained for purified ROS and 293S rhodopsins at 2, 4, 6, 8, and 10 nM concentrations.

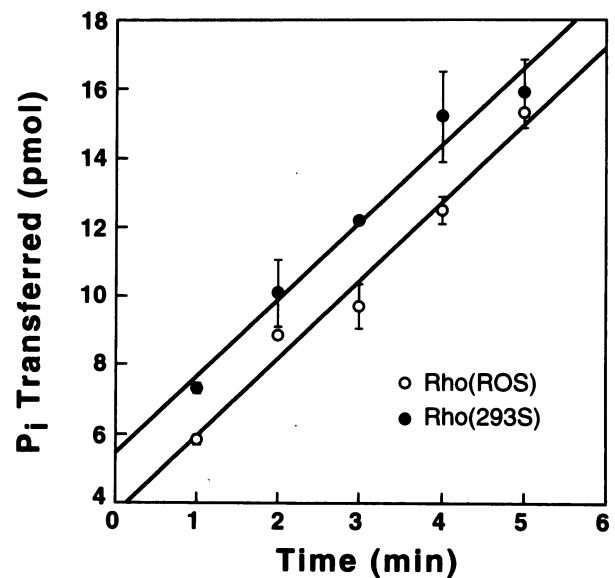


FIG. 5. Rates of phosphorylation of purified ROS and 293S rhodopsins by rhodopsin kinase.

in buffer B. Difference spectrum analysis showed a rhodopsin content of 1.37 mg in the total solubilized extract (20 ml), which was applied to a rho 1D4-Sepharose and purification carried out as described in *Materials and Methods*. Successive washings and elutions with different buffers were as shown in Fig. 2. Selected fractions were examined by SDS/PAGE, and the proteins were visualized both by silver staining (Inset A) and by immunoblotting (Inset B).

As seen both in the elution profile and SDS/PAGE analysis, rhodopsin eluted sharply in three fractions (59–61, all with A_{280}/A_{500} ratios between 1.6–1.73 (1.1 mg) and with a total recovery of 1.25 mg (91% of estimated expressed amount).

Characterization of 293S Rhodopsin. The UV/visible absorption spectra. The spectra, in the dark (A_{280}/A_{500}); 1.65 and after photobleaching, are shown in Fig. 3. These are identical to those for rhodopsin purified from ROS. Furthermore, the rate of metarhodopsin II decay as shown in Fig. 3 Inset ($T_{1/2}$, 15 min, 20°C) was indistinguishable from that of ROS rhodopsin (14).

Transducin activation by 293S rhodopsin. Initial rates of activation of transducin were measured (at 2–10 nM concentrations) by $G_T(\alpha):[\gamma\text{-}^{35}\text{S}]\text{GTP}$ complex formation. The results are shown in Fig. 4. Regression analysis performed on the data sets of ROS and 293S rhodopsin over five rhodopsin concentrations shows that the averaged initial rate values are essentially identical.

Light-dependent phosphorylation of 293S rhodopsin by rhodopsin kinase. Rates of phosphorylation of 293S rhodopsin and ROS rhodopsin were measured after illumination. The initial rates of phosphate incorporation, as determined by the slope (Fig. 5), were similar for both rhodopsins.

SDS/PAGE analysis and glycosylation profile of 293S rhodopsin. As shown above (Fig. 2) in SDS/PAGE analysis of fractions, purified 293S rhodopsin migrates as a smear and much slower than ROS rhodopsin (Fig. 6).

Preparations of ROS rhodopsin and 293S rhodopsin were digested with PNGase-F and the products analyzed as in Fig. 6. All three deglycosylated rhodopsins migrated identically (lanes 3, 5, and 7) with an apparent molecular mass of about 30 kDa. The slower bands observed in every case with identical mobility probably correspond to the dimers of the deglycosylated rhodopsins. Therefore, differences in mobilities of the three rhodopsins result from different *N*-glycosylation patterns.

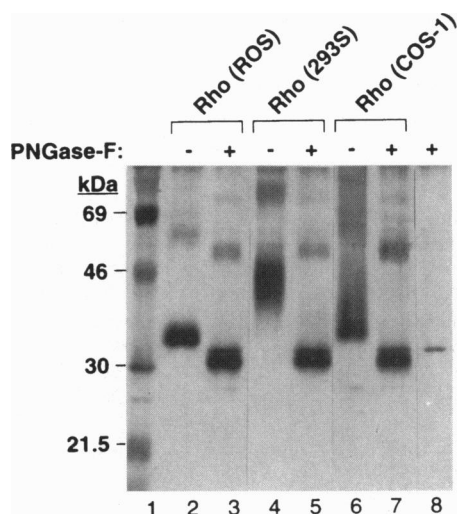


FIG. 6. Action of the deglycosylase, PNGase-F, on rhodopsins from different sources. The conditions for PNGase-F treatment are described in *Materials and Methods*. The analysis was by SDS/PAGE (10% gel), and visualization was by silver staining.

Cell Lines Expressing Rhodopsin Cytoplasmic Loop Mutants and Preparation of the Mutant Proteins. Using the strategy described above, the construction of stable cell lines expressing the following four rhodopsin cytoplasmic mutants (22) used for studies of rhodopsin–rhodopsin kinase interaction (R.L.T., C. Creuzenet, P.J.R., and H.G.K., unpublished work) was undertaken. The mutants contained: (i) deletion of the sequence Q237–E249, (ii) replacement of the amino acids at positions 140–152 with GTEGPNFYVPPTS, (iii) deletion of the sequence M143–E150, and (iv) replacement of the amino acids at positions 235–250 with TSLHGYSVTGPTG-SNL. The cell lines constructed produced these mutants in the range of 0.3–1 mg/liter of saturated cultures. For example, the cell line expressing mutant i accumulated the protein at a level of approximately 0.5 mg/liter. The mutant rhodopsin was purified from the cell culture as shown in Fig. 7, the procedure used and the results being essentially identical to those shown

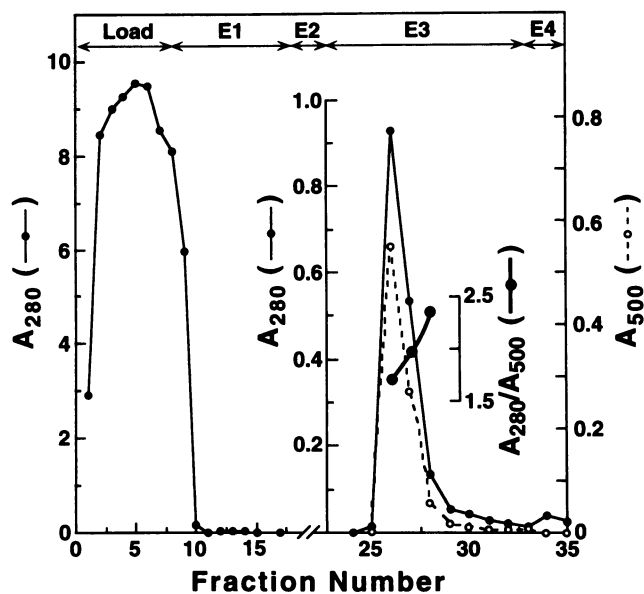


FIG. 7. Purification of rhodopsin mutant i expressed in the 293S cell line in a 1-liter starting culture. The conditions for immunoaffinity chromatography were identical to those described in Fig. 2. Recovery of purified mutant rhodopsin from the column was 401 μ g (89%) of the total (450 μ g) applied.

in Fig. 2 for wild-type rhodopsin. In Fig. 7, the majority of the mutant i rhodopsin eluted at the expected bed volume at a concentration of 12.3 μ M. The recovery from an estimated amount of 450 μ g of rhodopsin applied to the Sepharose column was 401 μ g (89%).

DISCUSSION

Methods currently available for expression of opsin genes are severely limited by the low yields of the functional proteins that they afford. Consequently, a variety of structure–function studies on rhodopsin, chemical and biophysical, as well as the use of potentially interesting mutants for crystallization, so far have been out of reach. Considerable effort has indeed been expended in a number of laboratories on investigating different expression systems for the preparation of opsin and its mutants. Expression in *E. coli* has not been encouraging (S. Karnik and P. Loewen, unpublished work in this laboratory). The yeast system, *S. cerevisiae*, as well as the methylotrophic *pichia pastoris*, have been investigated recently (1), but have not resulted in any significant advance for larger scale preparation of fully functional protein. The frequently used baculovirus-based expression system has been investigated by De-Grip and colleagues (23–25) and in this laboratory (S. Kaushal, unpublished work). In our view, this system has not resulted in any improvement in the availability of fully functional post-translationally processed protein that can be reliably used in signal transduction studies.

The mammalian stable cell lines now developed have enabled the preparation of rhodopsin and several of its mutants in amounts much larger than those previously thought practical. Furthermore, there is the efficient single-step purification procedure that results in homogeneous proteins in concentrated form. The applicability of these methods should be further extended by straightforward scale-up of the entire procedure for larger amounts of a variety of mutants that will be needed in future studies. A particularly critical advantage of the mammalian systems resides in the likelihood that they will perform specific post-translational modifications required for correct folding and function.

One property of the expressed proteins now characterized that became evident was the difference in electrophoretic mobility from those previously observed for ROS or COS cell produced rhodopsins. This was clearly the result of different *N*-glycosylation characteristics of the mammalian cell lines. However, stringent analysis of the expressed wild-type rhodopsin produced by all the methods available at present revealed no difference in function from ROS rhodopsin.

In conclusion, progress has been made in high level opsin gene expression and efficient purification of rhodopsin. The methods described should enable convenient scale-up of production and allow the preparation of a variety of mutants of interest in structure–function studies of rhodopsin.

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