lecular Structure in Biology, Vol. I, Ribonuclease S (Phillips, D. C., & Richards, F. M., Eds.) Clarendon, Oxford.
Saenger, W. (1973) *Angew. Chem., Int. Ed. Engl. 12*, 591–601.
Sheriff, S. (1987) *J. Appl. Crystallogr. 20*, 55–57.
Svensson, L. A., Sjölin, L., Gilliland, G., Finzel, B. C., & Wlodawer, A. (1987) *Proteins 1*, 370–375.
Ward, K. B., Wishner, B. C., Lattman, E. E., & Love, M. E. (1975) *J. Mol. Biol. 180*, 301–329.
Williams, R. L., Greene, S. M., & McPherson, A. (1987) *J. Biol. Chem. 262*, 16020–16031.
Wlodawer, A. (1985) in *Biological Macromolecules and Assemblies*, Vol. II, Nucleic Acids and Interactive Proteins (Jurnak & McPherson, Eds.) pp 395–439, Wiley, New York.
Wlodawer, A., & Sjölin, L. (1983) *Biochemistry 22*, 2720–2727.
Wlodawer, A., Svensson, L. A., Sjölin, L., & Gilliland, G. L. (1988) *Biochemistry 27*, 2705–2717.

Determinants of Visual Pigment Absorbance: Role of Charged Amino Acids in the Putative Transmembrane Segments†

Jeremy Nathans

Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, and Department of Neuroscience, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

Received August 14, 1989; Revised Manuscript Received September 14, 1989

ABSTRACT: I have investigated the effect on bovine rhodopsin's absorbance spectrum of charged amino acid changes in the putative membrane-spanning regions. A total of 14 site-directed mutants were constructed at 6 amino acid positions: 83, 86, 122, 134, 135, and 211. Two of these positions are occupied by charged amino acids that are conserved in all four human visual pigments (positions 134 and 135). In the four variable positions, single and double mutants were constructed to reproduce the intramembrane distribution of charged amino acids predicted for each human cone pigment. Following solubilization in digitonin and reconstitution with 11-*cis*-retinal, the photobleaching difference spectrum of each pigment was determined in the presence of hydroxylamine. The absorbance spectra of the mutant pigments are all surprisingly close to that of native bovine rhodopsin (λ_{max} = 498 nm), ruling out a significant role for these residues in spectral tuning.

Visual pigments are the light-absorbing proteins in the retina responsible for initiating visual excitation. Each consists of a protein, opsin, bound via a protonated Schiff's base to a small chromophore, 11-*cis*-retinal (or in some instances a closely related retinal). The absorbance spectra of a large number of visual pigments have been determined, and these spectra have in common a broad bell shape, closely resembling the shape of a protonated Schiff's base of retinal free in solution. They differ from one another and from the free chromophore in their positions along the wavelength axis: points of maximal absorbance have been found throughout the visible range, from far-red to near-ultraviolet. Presumably, amino acid sequence differences among the visual pigments determine their distinctive absorbance spectra.

The experiments reported here are aimed at determining the role of particular amino acids in spectral tuning. Recently, the amino acid sequences of a number of visual pigments have been determined, including bovine (Ovchinnikov et al., 1983; Hargrave et al., 1983; Nathans & Hogness, 1983), chicken (Takao et al., 1988), human (Nathans & Hogness, 1984), mouse (Baehr et al., 1988), octopus (Ovchinnikov et al., 1988), and *Drosophila* (O'Tousa et al., 1985; Zuker et al., 1985, 1987; Cowman et al., 1986; Montell et al., 1987) rhodopsins and the three human cone pigments (Nathans et al., 1986). These sequences define a family of homologous proteins and provide a data base to guide future experiments. It is reasonable to suppose that highly conserved properties such as efficient isomerization, transducin activation, and phosphorylation will be reflected in the conservation of single amino acids or protein domains. Conversely, those properties that differ between pigments, for example, the absorbance spectrum, will be reflected in protein sequence differences. This simple approach has been used effectively in many systems. To cite just one example, in the study of immunoglobulins, the regions which form the ligand binding pocket were correctly predicted from sequence comparisons before they were visualized by X-ray crystallographic studies (Wu & Kabat, 1970; Amzel et al., 1974).

The starting point for the experiments presented here is the spectral absorbance and amino acid sequence data for the four human visual pigments. Human rhodopsin, which mediates vision in dim light, peaks at 493–497 nm (Crescitelli & Dartnall, 1953; Wald & Brown, 1958); the three human cone pigments, which mediate color vision, peak at approximately 420, 530, and 560 nm [referred to as blue, green, and red pigments, respectively (Dartnall et al., 1983)]. Comparison of the red and green pigment amino acid sequences shows 96% identity whereas all other pairwise comparisons show approximately 40% identity (Nathans et al., 1986). The sequence differences define candidate amino acids that may be involved in spectral tuning.

All visual pigment sequences reveal seven predominantly hydrophobic stretches of amino acids. These are presumed to form a bundle of seven membrane-spanning α-helical segments, an arrangement consistent with protein modification experiments using water-soluble probes [see Nathans (1987) for a recent review]. 11-*cis*-Retinal is covalently attached to lysine-296 (in the bovine rhodopsin numbering system) in the center of the seventh hydrophobic stretch (Bownds, 1967;

† This research was supported by the National Eye Institute (NIH) and the Howard Hughes Medical Institute.

0006-2960/90/0429-937$02.50/0

Wang et al., 1980). Rhodopsin's polarized absorbance in oriented rod outer segments indicates that 11-*cis*-retinal lies parallel to the plane of the bilayer (Chabre et al., 1982). Moreover, fluorescence energy-transfer experiments indicate that the chromophore is close to the center of the bilayer (Thomas & Stryer, 1982), consistent with its site of covalent attachment. These and other experiments suggest that the seven membrane-spanning segments form a ring around a central retinal binding pocket. This model predicts that amino acids in several or all of these segments may come into close proximity to the chromophore. Most likely, amino acids that protrude beyond the membrane do not contact the chromophore.

Over the past 30 years, several chemically plausible mechanisms have been considered to explain spectral tuning. All incorporate the basic observation that photoexcitation of retinal leads to a significantly increased delocalization of π-electrons (Kropf & Hubbard, 1958; Mathies & Stryer, 1976). Any interactions that favor delocalization will selectively stabilize the excited state and lead to a red shift (i.e., a smaller energy difference between ground and excited states). Conversely, any interaction that decreases delocalization will lead to a blue shift (i.e., a larger energy difference between ground and excited states). The proposed mechanisms include (1) twisting about single bonds, leading to decreased delocalization (Blatz & Liebman, 1973), (2) twisting about double bonds, leading to increased delocalization [discussed in Honig et al. (1976)], (3) increasing the distance between the protonated Schiff's base and its presumptive counterion, leading to increased delocalization (Blatz et al., 1972), (4) placing polarizable groups in close proximity to the chromophore (Irving et al., 1969, 1970), thus stabilizing the excited state by compensatory electronic movements, and (5) placing one or more anions along the retinal chain—the point charge model—leading to increased delocalization (Kropf & Hubbard, 1958; Honig et al., 1976, 1979). Mechanisms 3 and 5 predict the existence of critically placed charged residues in the retinal binding pocket.

To test the role of charged amino acids in spectral tuning, we constructed and studied site-directed mutants of bovine rhodopsin. One set of mutants resembles the human cone pigments with respect to the distribution of charged residues in the putative transmembrane segments. A second set of mutants carries alterations in charged amino acids that are common to all human visual pigments. (Throughout this paper, the term "charged" will be used to refer to those amino acid residues that are most commonly ionized—aspartate, glutamate, lysine, arginine, and histidine. The possibility exists, however, that one or more of these residues may not be in its ionized state in rhodopsin.)

Materials and Methods

Plasmids and Cells. The rhodopsin expression plasmid pCIS-cRho consists of a pUC118 derivative carrying a human cytomegalovirus immediate early enhancer and promotor, an intron, the bovine rhodopsin coding region (Nathans & Hogness, 1983), an SV40 polyadenylation site, and the SV40 origin of DNA replication (Eaton et al., 1986; Pritchett et al., 1988; Nathans et al., 1989). 293S, a suspension-adapted variant of a human embryonic kidney cell line [ATCC CRL 1573; grown in 10% fetal calf serum (Hyclone), 50:50 F12/DMEM with low glucose (Gibco) at 37 °C in a 5% CO_2 atmosphere], was used as the recipient for transient transfection. In a typical experiment, 100 μg of a pCIS-cRho mutant plasmid and 10 μg of pRSV-TAg (an SV40 T-antigen expression plasmid) were coprecipitated onto 20 10-cm diameter plates of 293S cells by the calcium phosphate method (Gorman, 1985).

In Vitro Mutagenesis. Mutagenic oligonucleotides were used to prime DNA synthesis on a single-stranded pCIS-cRho template as described (Carter, 1987). Covalently closed double-stranded circular products were gel purified and transfected into an *Escherichia coli* mutant deficient in the mismatch repair gene *mutL* (Glickman & Radman, 1980). After overnight growth in liquid culture, plasmid DNA was harvested from the pool of *mutL* transformants; the rhodopsin-coding region was excised, gel purified, ligated to the parental pCIS plasmid, and introduced into wild-type *E. coli*. Single colonies harboring mutant plasmids were identified by colony hybridization using the mutagenic oligonucleotides as probes. For each mutant, the entire rhodopsin-coding region was sequenced on one strand by the dideoxy method, both to confirm the predicted nucleotide changes and to rule out spurious ones.

Membrane Preparation and Joining to 11-cis-Retinal. Solutions for membrane preparation were chilled on ice, and all centrifugation steps were performed at 4 °C. Sixty hours after transfection, the cells were collected by vigorously washing the plates with phosphate-buffered saline (PBS) containing 5 mM EDTA. The cells were centrifuged at 1000*g* for 10 min, washed once with 20 mL of PBS, and resuspended in 10 mL of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, 250 mM sucrose, and 0.2 mM PMSF. The cell suspension was homogenized for 30 s in a Polytron homogenizer (Brinkmann) at a setting of 5.5, diluted to 20 mL with the same buffer, and then layered on top of two 5-mL 1.15 M sucrose cushions containing 0.1 M sodium phosphate, pH 6.5, and 1 mM EDTA. The homogenate was centrifuged in a swinging-bucket rotor (SW40) at 105000*g* for 30 min. Membranes were collected from the interface between the homogenization buffer and the sucrose cushion, diluted with 8 volumes of 0.1 M sodium phosphate, pH 6.5, and 1 mM EDTA, and centrifuged in a swinging-bucket rotor at 105000*g* for 30 min. The resulting membrane pellet was solubilized in 0.3 mL of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, and 5% digitonin (Kodak) at 23 °C.

All manipulations involving 11-*cis*-retinal were performed under dim red light. 11-*cis*-Retinal (stored dry in the dark under argon at −80 °C) was first dissolved in ethanol to a concentration of 4×10^{-3} M and then diluted with 20 volumes of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, and 5% digitonin; 0.1 mL of the aqueous 11-*cis*-retinal solution was added to the solubilized preparation and incubated at 23 °C for 2 h in the dark. The reaction was terminated by the addition of 0.05 mL of 0.6 M NH_2OH (adjusted to pH 6.0 with NaOH) and the sample incubated for an additional 30 min at 23 °C (Hubbard et al., 1971).

Absorbance Spectra. Before measurement of the absorbance spectrum, samples were centrifuged at 11 000 rpm in an Eppendorf microfuge for 5 min. Spectra were obtained on the supernatant before and after 10 min of photobleaching with a 15-W incandescent bulb placed behind a glass shield approximately 10 cm from the cuvette. Spectra were recorded and analyzed on a Kontron Instruments Uvikon spectrophotometer.

Protein Blots. Membrane samples used for spectral absorbance measurements were diluted with 5 volumes of SDS sample buffer, electrophoresed on an SDS/12% polyacrylamide gel, and electroblotted onto nitrocellulose (Burnette, 1981). Opsin was detected by using a mouse monoclonal anti-bovine rhodopsin antibody (B6-30; a kind gift of Dr. Paul

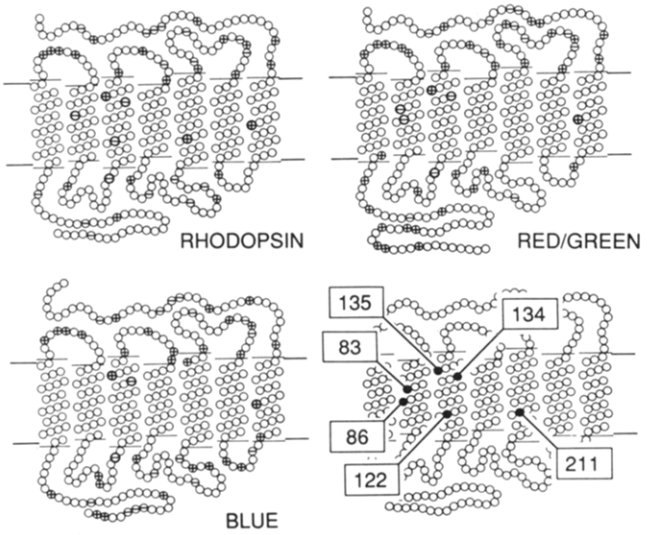

FIGURE 1: Charged amino acids in human and bovine visual pigments. The transmembrane topography is based on the model of Hargrave et al. (1983). Arginine, lysine, and histidine residues are indicated by (+); glutamate and aspartate by (−). Mutated residues are indicated and are numbered by using the bovine rhodopsin numbering system.

Table I: Amino Acids in Bovine Rhodopsin and the Four Human Visual Pigments at the Six Putative Intramembrane Positions[a]

amino acid position	human/bovine rhodopsin	human blue	human red/green
83	Asp	Gly	Asp
86	Met	Leu	Glu
122	Glu	Leu	Ile
134	Glu	Glu	Glu
135	Arg	Arg	Arg
211	His	Cys	Cys

[a] The bovine rhodopsin numbering system is used.

Hargrave) and an alkaline phosphatase conjugated rabbit anti-mouse second antibody (Cappel).

Results

Choice of Mutants. A comparison of the charged amino acids in the four human visual pigments is shown in Figure 1. The transmembrane models are based on the one proposed by Hargrave et al. (1983) for bovine rhodopsin; similar models have been proposed by Ovchinnikov et al. (1982) and Nathans and Hogness (1983). If we include histidine as a potentially charged residue and exclude lysine[296] to which 11-*cis*-retinal binds, then there are six positions of interest in the putative transmembrane segments at which charged residues appear. Two of these positions, 134 and 135, are occupied by invariant amino acids. In the putative transmembrane regions, the blue pigment has only the conserved charges, the red and green pigments have two additional charged residues, and rhodopsin has three additional charged residues, one of which (aspartate[83]) is shared with the red and green pigments. Table I lists the amino acids at these six positions for each of the pigments. (Numbering of amino acids follows the bovine rhodopsin sequence.)

In the experiments reported here, I have chosen to study bovine rather than human rhodopsin because it is the better characterized of the two visual pigments. I would anticipate similar results with human rhodopsin given that the two are 94% identical at the amino acid level, have identical charged residues, and have absorbance maxima within several nanometers of one another (Crescitelli & Dartnall, 1953; Wald & Brown, 1958). Nine site-directed mutants were constructed

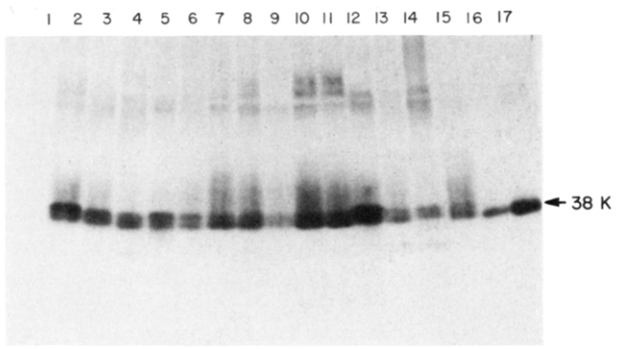

FIGURE 2: Protein blots of expressed rhodopsin mutants. Each lane of an SDS/12% polyacrylamide gel was loaded with 2% of the sample used for spectrophotometry and visualized with a monoclonal mouse anti-bovine rhodopsin primary antibody and alkaline phosphatase conjugated rabbit anti-mouse secondary antibody. Lanes: 1, control transfection with pCIS carrying an irrelevant cDNA; 2, D83G; 3, D83N; 4, M86E; 5, E122Q; 6, E122I; 7, E134L; 8, E134Q; 9, R135L; 10, H211C; 11, H211F; 12, E122I D83G; 13, E122I M86E; 14, E134L R135L; 15, E134R R135E; 16, 40 ng of bovine rhodopsin from rod outer segments; 17, 200 ng of bovine rhodopsin from rod outer segments.

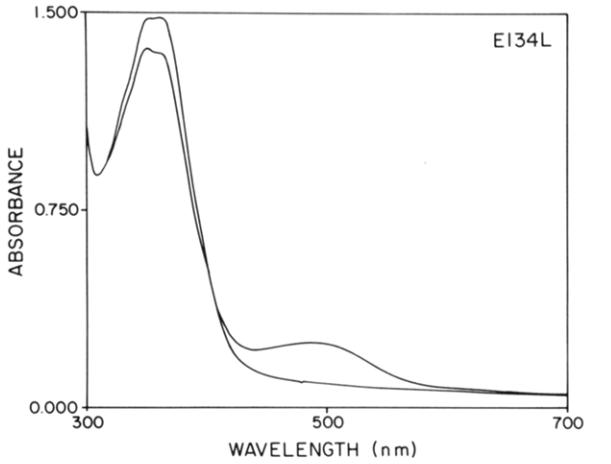

FIGURE 3: Typical absorbance spectra before and after photobleaching of a rhodopsin mutant expressed in 293S cells. The upper curve in the visible region is before photobleaching. The large absorbance in the ultraviolet region is due to retinal oxime.

to produce the cone pigment charge distributions at the four variable positions. Each of the single amino acid substitutions were constructed: D83G, M86E, E122I, and H211C. (Mutants are referred to by the single-letter amino acid designation of the wild-type residue followed by its position number in the polypeptide chain followed by the single-letter amino acid designation of the introduced residue; e.g., D83G refers to the substitution of aspartate[83] by glycine.) In addition, single-substitution mutants were constructed that minimize structural perturbations not related to charge (D83N, E122Q, and H211F). Two double mutants were constructed by sequential mutagenesis to produce the charge distributions corresponding to the blue pigment (E122I D83G) and the red and green pigments (E122I M86E). Five mutants were constructed in the conserved glutamate[134]-arginine[135] pair: each was changed to a neutral hydrophobic residue (E134L and R135L), both were changed (E134L R135L), glutamate was changed to the most similar uncharged residue, glutamine (E134Q), and the two were swapped (E134R R135E).

Expression. cDNA encoding each mutant pigment was inserted into a plasmid vector containing a human cytomegalovirus immediate early enhancer/promoter and an SV40 origin of DNA replication (Eaton et al., 1986; Pritchett et al., 1988). Transient cotransfection (Gorman, 1985) of a human

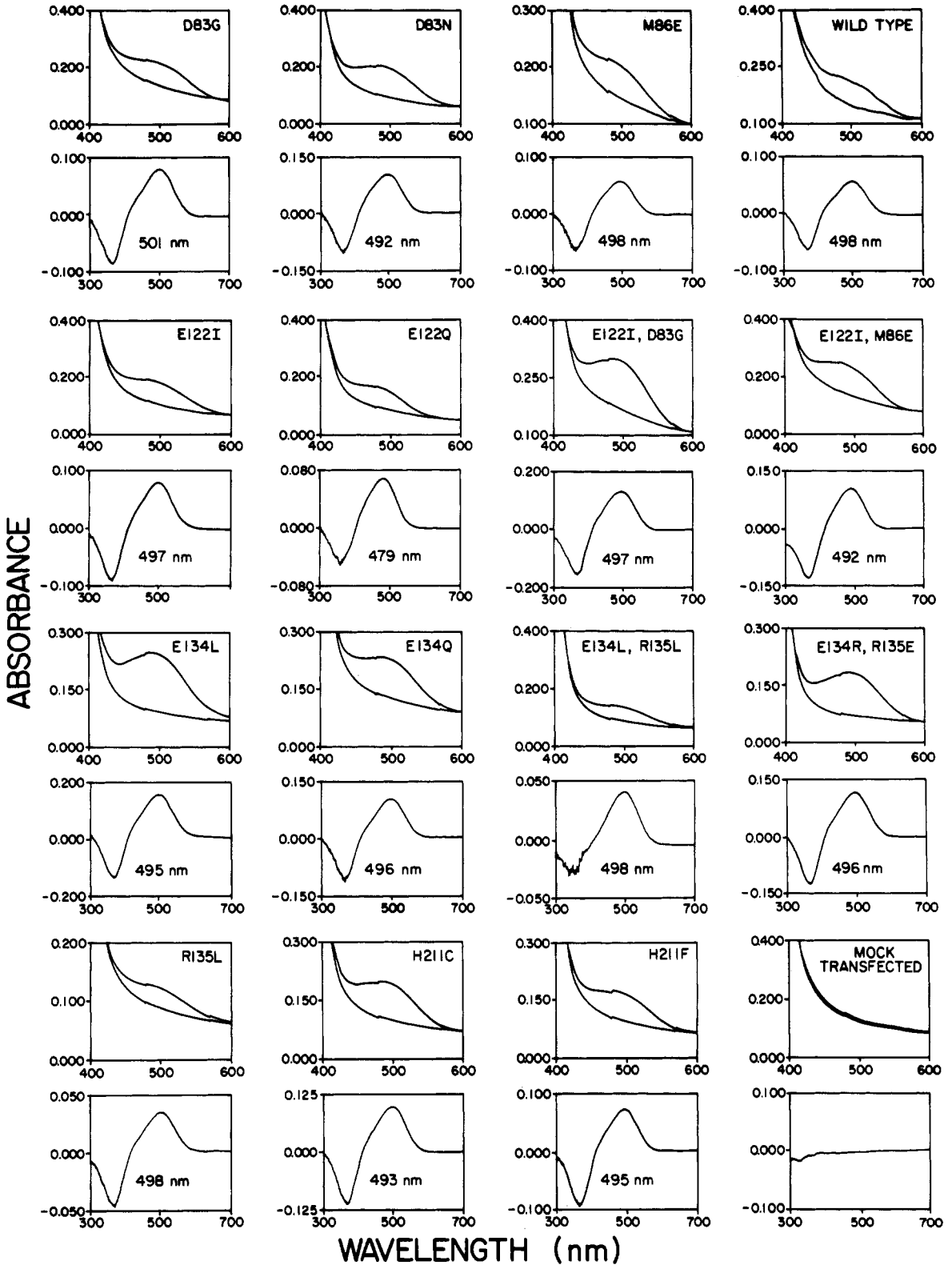

FIGURE 4: Absorbance spectra before and after photobleaching (upper panel) and difference spectra (lower panel) of expressed mutant rhodopsins, wild-type bovine rhodopsin, and mock-transfected 293S cells.

embryonic kidney cell line, 293S, with the expression plasmid and a plasmid directing expression of the SV40 large T-antigen causes the expression plasmid to replicate to high levels. At 60 h after transfection, approximately 2 μg of opsin accumulated per 10^7 cells, as determined spectrophotometrically following reconstitution (see below) and by protein blotting (Figure 2). This level is comparable to that seen in stable cell lines as described earlier (Nathans et al., 1989) and corresponds to an average of 3×10^6 molecules per cell.

Reconstitution with 11-cis-Retinal and Measurement of Spectral Absorbance. For reconstitution with 11-*cis*-retinal, total cell membranes were harvested, solubilized in digitonin, and incubated in the dark for 2 h with 2×10^{-8} mol of 11-*cis*-retinal. Hydroxylamine was then added to 50 mM to convert free retinal to retinal oxime (Hubbard et al., 1971). Spectra were recorded before and after photobleaching (Figure 3). Upon incubation with 11-*cis*-retinal, all of the mutant opsins produce photolabile pigments with absorbance spectra

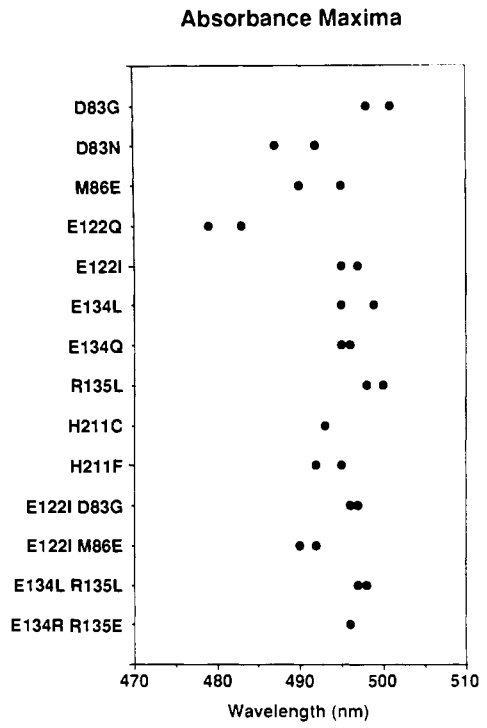

FIGURE 5: Difference spectra absorbance maxima from two independent experiments for each rhodopsin mutant. In two experiments, expression of a wild-type rhodopsin cDNA produced a pigment with absorbance maxima at 497 and 498 nm.

similar in shape to that of rhodopsin (Figure 4). All of the pigments are stable in the presence of 50 mM hydroxylamine for 30 min at room temperature. Incubation of 11-*cis*-retinal with membranes from mock-transfected cells does not produce this absorbance (Figure 4).

The photobleaching difference spectra are highly reproducible as judged by a comparison of two independent transfection and reconstitution experiments for each pigment (Figure 5). Endogenous cellular constituents give only trace absorbance in the visible range, none of which appears to be photolabile. Pigment yields varied from 0.025 to 0.155 ODU, with a mean of 0.074 ODU. If we assume that the mutant pigments have nearly the same molar extinction coefficient as rhodopsin, then the yields calculated from the absorbance spectra are within a factor of 2 of the yields estimated from protein blots. The absorbance maxima showed a mean variation in two trials of 2.4 nm, the greatest spread being 5 nm.

The absorbance maximum for wild-type rhodopsin produced in this expression system is 498 nm (Nathans et al., 1989), identical with that for rhodopsin isolated from bovine retinas. The absorbance maximum of each mutant pigment will be taken as the average of the two experimental determinations. Substitution of aspartate[83] with glycine or asparagine leads to shifts of +1.5 and −8.5 nm, respectively; substitution of glutamate[122] with isoleucine or glutamine leads to shifts of −2 and −17 nm, respectively; substitution of aspartate[83] with glycine together with substitution of glutamate[122] with isoleucine leads to a shift of −1.5 nm; and substitution of histidine[211] with phenylalanine or cysteine leads to shifts of −4.5 and −5 nm, respectively. These alterations mimic the charge distribution seen in the human blue cone pigment, the absorbance maximum of which is shifted −68 ± 10 nm with respect to rhodopsin.

Substitution of methionine[86] with glutamate leads to a shift of −5.5 nm, and substitution of methionine[86] with glutamate together with substitution of glutamate[122] with isoleucine leads to a shift of −7 nm. These alterations, together with E122Q, E122I, H211F, and H211C described above, mimic the charge distribution seen in the human red and green pigments, the absorbance maxima of which are shifted +32 and +62, respectively, relative to rhodopsin.

Substitution of the two conserved residues, glutamate[134] and arginine[135], results in almost no spectral shifts; substitution of arginine[135] with leucine leads to a shift of +1 nm; substitution of glutamate[134] with leucine or glutamate leads to shifts of −1 and −2.5 nm, respectively; substitution of both glutamate[134] and arginine[135] with leucine leads to a shift of −0.5 nm; and swapping glutamate[134] and arginine[135] leads to a shift of −2 nm.

Discussion

Charged amino acids have been hypothesized to play two distinct roles in spectral tuning. One negatively charged residue is presumed to be near the protonated Schiff's base nitrogen and to serve as its counterion (Honig et al., 1976). A second negatively charged residue is postulated to lie near the polyene chain to stabilize excited-state resonance structures (the point-change model; Kropf & Hubbard, 1958; Honig et al., 1976, 1979). In bovine rhodopsin, the second negatively charged residue is hypothesized to be near C-13 of 11-*cis*-retinal based on the spectral shifts observed when dehydroretinal analogues are used to reconstitute the pigment (Koutalos et al., 1989). Analogous experiments with chicken iodopsin (λ_{max} = 562 nm) have led to the hypothesis that a negatively charged residue in that pigment is near the β-ionone ring (Chen et al., 1989). Substitution by a neutral residue of the putative Schiff's base counterion should increase π-electron delocalization and produce a red shift, whereas substitution by a neutral residue of the putative point charge near the polyene chain should decrease π-electron delocalization and produce a blue shift.

The surprising result of the experiments reported here is that charge alterations at each of the six candidate positions in the putative transmembrane segments produce absorbance shifts far smaller than those seen in the cone pigments. Mutant E122Q produced the largest spectral shift, −17 nm. However, a more drastic substitution at that same position, E122I, produced only a −2-nm shift, implying that glutamate[122] is not intimately involved in spectral tuning.

This result rules out a significant role for these residues in spectral tuning. By contrast, had these mutations conferred large spectral shifts, the data would be open to two interpretations: either the altered residues interact directly with the chromophore or they influence the conformation of a part of the protein that does interact directly. In general, these two interpretations would not be easily resolvable without a high-resolution three-dimensional structure.

One interpretation of these results is that charged amino acids make little electronically significant contact with the chromophore. The counterion may correspond to a solvent ion, or, alternatively, the protonated Schiff's base may be stabilized by a surrounding shell of polar residues analogous to the hydration sphere that surrounds ions in solution. The latter arrangement would be expected to contribute to the red shift observed in comparing a chloride salt of the protonated Schiff's base of retinal in methanol (λ_{max} = 440 nm; Blatz et al., 1972) to bovine rhodopsin (λ_{max} = 498 nm). With respect to the absorbance difference that distinguishes different pigments, this interpretation would imply that visual pigments use tuning mechanisms other than charge mechanisms. A similar conclusion has been drawn for bacteriorhodopsin based on recent in vitro mutagenesis experiments (Mogi et al., 1988). This is clearly the case for the absorbance difference between

human red and green cone pigments. Although their absorbance maxima differ by 30 nm, their amino acid sequences differ at only 15 positions, none of which are charged.

A second interpretation of these results is that one or more charged amino acids other than the ones I have mutated interact with the Schiff's base and/or perturb the polyene chain. This would imply that the working model of transmembrane topography needs to be revised. This interpretation can be tested directly by mutating every charged amino acid in rhodopsin.

ACKNOWLEDGMENTS

I thank Dr. Axel Ullrich (Genentech) in whose laboratory this work began; Dr. Cornelia Gorman, Dr. Arthur Levinson, and Robert Kline (Genentech) for advice on tissue culture expression systems and for making available the CIS vector and 293S cell line used in this work; Dr. Lubert Stryer (Stanford University) and the Hoffmann-La Roche Co. for gifts of 11-*cis*-retinal; Dr. Paul Hargrave (University of Florida) for the gift of monoclonal anti-bovine rhodopsin antibody; Dr. Isabel Chiu, Shannath Merbs, and Yanshu Wang for advice; Drs. Isabel Chiu, Charles Weitz, and Donald Zack for helpful comments on the manuscript; and Teri Chase for assistance in preparing the manuscript.

Registry No. Asp, 56-84-8; Glu, 56-86-0; Arg, 74-79-3; His, 71-00-1; Lys, 56-87-1.

REFERENCES

Amzel, L. M., Poljak, R. J., Saul, F., Vargo, J. M., & Richards, F. F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1427–1430.

Baehr, W., Falk, J. D., Bugra, K., Triantafyllos, J. T., & McGinnis, J. F. (1988) *FEBS Lett. 238*, 253–256.

Blatz, P. E., & Liebman, P. A. (1973) *Exp. Eye Res. 17*, 573–580.

Blatz, P. E., Mohler, J., & Navangul, H. (1972) *Biochemistry 11*, 848–855.

Bownds, D. (1967) *Nature 216*, 1178–1181.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Carter, P. (1987) *Methods Enzymol. 154*, 382–403.

Chabre, M., Breton, J., Michel-Villaz, M., & Saibil, H. (1982) *Methods Enzymol. 81*, 605–616.

Chen, J. G., Nakamura, T., Ebrey, T. G., Ok, H., Konno, K., Derguini, F., Nakanishi, K., & Honig, B. (1989) *Biophys. J. 55*, 725–729.

Cowman, A. C., Zuker, C. S., & Rubin, G. M. (1986) *Cell 44*, 705–710.

Crescitelli, F., & Dartnall, H. J. A. (1953) *Nature 172*, 195–197.

Dartnall, H. J. A., Bowmaker, J. K., & Mollon, J. D. (1983) *Proc. R. Soc. London, B 220*, 115–130.

Eaton, D. L., Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Lawn, R. M., Vehar, G. A., & Gorman, C. (1986) *Biochemistry 25*, 8343–8347.

Glickman, B. W., & Radman, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1063–1067.

Gorman, C. (1985) in *DNA Cloning* (Glover, D. M., Ed.) Vol. II, pp 143–190, IRL Press, Oxford.

Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S. L., Mohana, Rao, J. K., & Argos, P. (1983) *Biophys. Struct. Mech. 9*, 235–244.

Honig, B., Greenberg, A., Dinur, V., & Ebrey, T. (1976) *Biochemistry 15*, 4593–4699.

Honig, B., Dinur, U., Nakanishi, K., Balough-Nair, V., Gawinowicz, M. A., Arnaboldi, M., & Motto, M. G. (1979) *J. Am. Chem. Soc. 101*, 7084–7086.

Hubbard, R., Brown, P. K., & Bownds, D. (1971) *Methods Enzymol. 18*, 615–653.

Irving, C. S., Byers, G. W., & Leermakers, P. A. (1969) *J. Am. Chem. Soc. 91*, 2141–2143.

Irving, C. S., Byers, G. W., & Leermakers, P. A. (1970) *Biochemistry 9*, 858–864.

Koutalos, Y., Ebrey, T. G., Tsuda, M., Odashima, K., Lien, T., Park, M. H., Shimizu, N., Derguini, F., Nakanishi, K., Gilson, H. R., & Honig, B. (1989) *Biochemistry 28*, 2732–2739.

Kropf, A., & Hubbard, R. (1958) *Ann. N.Y. Acad. Sci. 74*, 266–280.

Mathies, R., & Stryer, L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2169–2173.

Mogi, T., Stern, L. J., Merti, T., Chao, B. H., & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4148–4152.

Montell, C., Jones, K., Zuker, C., & Rubin, G. M. (1987) *J. Neurosci. 7*, 1558–1566.

Nathans, J. (1987) *Annu. Rev. Neurosci. 10*, 163–194.

Nathans, J., & Hogness, D. S. (1983) *Cell 34*, 807–814.

Nathans, J., & Hogness, D. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4851–4855.

Nathans, J., Thomas, D., & Hogness, D. S. (1986) *Science 232*, 193–202.

Nathans, J., Weitz, C. J., Agarwal, N., Nir, I., & Papermaster, D. S. (1989) *Vision Res. 29*, 907–914.

O'Tousa, J. E., Baehr, W., Martin, R. L., Hirsh, J., Pak, W. L., & Applebury, M. W. (1985) *Cell 40*, 839–850.

Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Artamonov, I. D., Bogachuk, A. S., Zolotarev, A. S., Eganyan, E. R., & Kostetskii, P. V. (1983) *Bioorg. Khim. 9*, 1331–1340.

Ovchinnikow, Yu. A., Abdulaev, A. S., Zolotarev, A. S., Artamov, I. D., Bespalov, I. A., Dergachev, A. E., & Tsuda, M. (1988) *FEBS Lett. 232*, 69–72.

Pritchett, D. B., Sontheimer, H., Gorman, C. M., Ketterman, H., Seeburg, P. H., & Schofield, P. R. (1988) *Science 242*, 1306–1308.

Takao, M., Yasui, A., & Tokunaga, F. (1988) *Vision Res. 28*, 471–480.

Thomas, D. D., & Stryer, L. (1982) *J. Mol. Biol. 154*, 145–157.

Wald, G., & Brown, P. K. (1958) *Science 127*, 222–226.

Wang, J. K., McDowell, J. H., & Hargrave, P. A. (1980) *Biochemistry 19*, 5111–5117.

Wu, T. T., & Kabat, E. A. (1970) *J. Exp. Med. 132*, 211–250.

Zuker, C. S., Cowman, A. F., & Rubin, G. M. (1985) *Cell 40*, 851–858.

Zuker, C. S., Montell, C., Jones, K., Laverty, T., & Rubin, G. M. (1987) *J. Neurosci. 7*, 1550–1557.