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Mutagenesis studies of rhodopsin phototransduction

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We employ the vertebrate visual proteins rhodopsin and transducin as a model system for structure-function studies on the molecular mechanisms of transmembrane signaling. These visual proteins are members of a super-family of related guanine nucleotide-binding regulatory proteins (G proteins) and G protein-coupled receptors. We are particularly interested in the structures of the retinal binding pockets of visual pigments and in identifying the structural domains of both rhodopsin and transducin involved in the G protein activation process.

AMINO ACID SUBSTITUIONS THAT CAUSE BATHOCHROMIC SPECTRAL SHIFTS IN BOVINE RHODOPSIN

Nearly all vertebrate visual pigments share a common chromophore, 11-*cis*-retinal. In humans, the differences in absorption maxima of the cone pigments that underlie human red-green color vision must result from differences in the amino acid sequences of the respective opsin proteins. Fifteen amino acid substitutions distinguish the human green pigment (λ_{max} = 530 nm) from the human red pigment (λ_{max} = 560 nm) (Nathans *et al.*, 1986). Three of these residues were suggested in a genetic analysis of eight primate visual pigments to produce this spectral difference of about 1000 cm^{-1} (Neitz *et al.*, 1991). The amino acid at each of these three positions in the rod pigment rhodopsin (λ_{max} = 500 nm), matches that of the green pigment (Table 1) (Nathans & Hogness, 1983; Nathans *et al.*, 1986). Therefore, it was postulated that the influence of these residues could be tested experimentally by substituting the amino acid residues of the red pigment into rhodopsin. A mutation resulting in a red shift in absorption maximum relative to rhodospin would indicate potential relevance in red-green spectral tuning.

We recently reported seven bovine rhodopsin mutants involving the three amino acid positions: three single substitutions, Ala 164 replaced by Ser (A164S), Phe 261 replaced by Tyr (F261Y), and Ala 269 replaced by Thr (A269T); three double substitutions; and one triple substitution (Chan *et al.*, 1992). Replacement of Ala 164 caused only a slight red shift effect (λ_{max} = 502 nm). However, replacement of Phe 261 or Ala 269 caused red-shifted λ_{max} values of 510 nm and 514 nm, respectively. The double replacement at both positions 261 and 269 simultaneously caused a red shift to 520 nm that was greater than either of the two substitutions alone but not strictly additive. Replacement at both positions 164 and 261 caused a red shift (λ_{max} = 512 nm) that was slightly greater than that of F261Y alone. Replacement at both positions 164 and 269 resulted in a λ_{max} value of 514 nm that was the same as that of the single substitution at position 269. Two of the three positions (261 and 269) in combination appear to account for the 775 cm^{-1} of the observed 1000 cm^{-1} difference between the human green and red pigments (Table 2). The triple mutant did not bind 11-*cis*-retinal to form a pigment. It is not known whether the triple mutant, if it could be induced to bind 11-*cis* -retinal, would display the full

1000 cm^{-1} red shift. However, the effects of all combinations of double replacements were qualitatively additive but not synergistic. For the triple mutant to account for the entire 1000 cm^{-1} shift, a synergistic effect would be required.

Table 1 Comparison of amino acids in various pigments at positions proposed to account for red-green spectral tuning*

bovine rhodopsin	human rhodopsin	human green	human red
Ala 164	Ala 164	Ala 180+	Ser 180
Phe 261	Phe 261	Phe 277	Tyr 277
Ala 269	Ala 269	Ala 285	Thr 285

*The numbering system shown is from previous reports of the deduced amino acid sequences of bovine rhodopsin (Nathans & Hogness, 1983), human rhodopsin (Nathans & Hogness, 1984), and human cone pigments (Nathans *et al.*, 1986).
+a genetic polymorphism was reported at this position that could potentially result in Ser at this position as well (Nathans *et al.*, 1986).

The most likely explanation for the observed red-shifted λ_{max} values is that a newly introduced hydroxyl-bearing amino acid residue can interact directly with the chromophore. However, it is possible that an individual amino acid replacement causes distant effects on the chromophore binding pocket. The effect of a mutation on absorption maximum may result from an indirect effect as well as a direct interaction. However, whereas blue-shifted λ_{max} values indicate a relative loss of chromophore-protein interactions, red-shifted λ_{max} values indicate an enhanced interaction. A mutant with a red-shifted λ_{max} value has a larger opsin shift than that normally observed in rhodopsin. Attributing an effect on absorption maximum to a specific amino acid-chromophore interaction is likely to be more valid in cases where a red shift rather than a blue shift is observed. A large number of rhodopsin mutants have been previously reported that cause blue-shifted absorption maxima (Nakayama & Khorana, 1990; Nathans, 1990a). No significant red-shifted mutants have been reported other than those involving the Schiff base counterion at position Glu 113 (Nathans, 1990b; Sakmar *et al.*, 1989; Sakmar *et al.*, 1991, Zhukovsky & Oprian, 1989).

Although residues in rhodopsin match those in the green pigment at the three positions tested, the rhodopsin and the green pigment are only about 70% homologous (Nathans *et al.*, 1986). Obviously the retinal binding pocket in rhodopsin is significantly different from that of the green pigment as demonstrated by the 1,125 cm^{-1} difference between their spectral peaks. However, at the three positions proposed from primary structure comparisions to account for red-green pigment spectral tuning, rhodopsin and the green pigment share the same residues. In addition, the design of this experiment involves testing a hypothesis by correlating mutagenesis with the appearance of a red-shifted absorption maximum (increase in opsin shift), and not with the loss of an existing retinal-protein interaction as indicated by a blue-shifted absorption maximum (decrease in opsin shift).

Neitz *et al.* (1991) hypothesized that additive effects of changes at amino acid positions 180, 277, and 285 should account for all shifts in spectra among a set of primate visual pigments (see Table 1 for a comparison of numbering systems in rhodopsin versus cone pigments) (Neitz *et al.*, 1991). They argued that the effects of changes at positions 180 and 285 were shifts of about 5 and 15.5 nm respectively and that the remaining 9- to 10-nm difference was produced by the substitution at position 277. We conclude that two of these residues (Tyr 277 and Thr 285) are primarily involved in spectral tuning that distinguishes red from green pigments, but that the effects of individual differences may not be strictly additive. For example, single substitutions in rhodopsin at position 261 (F261Y) and postion 269 (A269T) result in red shifts of 400 cm^{-1} and 550 cm^{-1}, respectively. However, in combination these two replacements cause a red shift of 775 cm^{-1}. Also, replacement at position 164 (A164S) results in a slight red shift (75 cm^{-1}). This effect was additive in combination with F261Y but

not in combination with A269T (Table 2).

Table 2 Rhodopsin mutants designed to mimic naturally occuring substitutions in green and red pigments

Mutation(s)	λ_{max} (nm)*	Shift from rho (cm^{-1})+
A164S	502	75
F261Y	510	400
A269T	514	550
F261Y/A269T	520	775
A164S/F261Y	512	475
A164S/A269T	514	550
A164S/F261Y/A269T	n.d.	-

Site-directed mutagenesis was performed using restriction fragment replacement in a synthetic gene (Oprian *et al.*, 1986). The altered genes were expressed in COS-1 cells and purified by an immunoaffinity procedure (Oprian *et al.*, 1987; Sakmar *et al.*, 1989).
* λ_{max} was determined from the peaks of photobleaching difference spectra. The precision is estimated to be +/- 2 nm. The λ_{max} of rhodopsin purified from COS cells was 500 nm.
+ λ_{max} shifts from that of rhodopsin are expressed in wavenumbers (cm^{-1}) to allow a direct comparison of energy differences. Values are rounded to the nearest 25 cm^{-1}. All shifts were to longer wavelengths (red shifts).
n.d. - the triple mutant did not bind 11-*cis*-retinal to form a pigment.

The red shift attributable to positions 277 and 285 in combination (775 cm^{-1}) is a significant fraction of the observed difference in absorption maxima between the human green and red pigments (1000 cm^{-1}). Other amino acid residues, including that at position 180, are likely to contribute to lesser degrees to account for the remaining 250 cm^{-1}. In rhodopsin, spectral tuning was shown not to be influenced by electrostatic interaction with carboxylates other that the counterion (Nathans, 1990a; Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989). A neutral chromophore binding pocket model in which dipole and hydrogen bonding interactions predominate has been proposed (Birge *et al.*, 1988; Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989). Since the mutations described in this report account for more than three quarters of the difference in absorption maxima between green and red pigments, a similar neutral chromophore binding pocket model is likely to apply to the green and red color pigments as well. A complete understanding of spectral tuning in the visual pigments will require detailed spectroscopic studies, including resonance Raman spectroscopy of mutant rhodopsins (Lin *et al.*, 1992) and cone pigments that have recently been expressed (Merbs & Nathans, 1992; Oprian *et al.*, 1991).

FLUORESCENCE STUDIES OF RHODOPSIN-TRANSDUCIN INTERACTIONS

Light-activated rhodopsin catalyzes guanine nucleotide exchange by transducin. We are interested in identifying specific domains of rhodopsin and transducin involved in binding and activation. It has previously been shown by flash photolysis studies of site-directed rhodopsin mutants that loop CD and loop EF of rhodopsin are involved in activation of bound transducin (Franke *et al.*, 1990). Other mutations prevent transducin binding. Recently, we have developed a spectrofluorimetric method designed to allow simultaneous illumination and excitation-emission fluorescence measurements of rhodopsin. Rhodopsin-catalyzed binding of GTP or a GTP analog to transducin results in a large increase in its intrinsic fluorescence. Mixtures of transducin and rhodopsin can be assayed by this method to determine the kinetic rate constants of their interaction and to evaluate the specific effects of mutations. Studies of a series of site-directed mutants of rhodopsin with alterations in their cytoplasmic domains are underway.

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