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Introduction of Hydroxyl-bearing Amino Acids Causes Bathochromic Spectral Shifts in Rhodopsin

AMINO ACID SUBSTITUTIONS RESPONSIBLE FOR RED-GREEN COLOR PIGMENT SPECTRAL TUNING*

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Comparisons of the deduced amino acid sequences of eight primate photopigment genes led to the proposal that three amino acid substitutions produce the approximately 1,000 cm^{-1} difference in the absorption maxima of human red and green pigments (Neitz, M., Neitz, J., and Jacobs, G. H. (1991) *Science* 252, 971–974). We tested this proposal by mutating these three residues in rhodopsin and evaluating the effects on spectral properties. Nonpolar residues normally present in rhodopsin and in the green pigment were substituted by hydroxyl-bearing residues normally present in the red pigment. Two of these substitutions (Phe-261 to Tyr or Ala-269 to Thr) caused significant red shifts in the absorption maxima of the resulting mutant pigments. A third substitution (Ala-164 to Ser) caused only a slight effect. Combinations of substitutions caused additive shifts in absorption maxima. A double mutant (Phe-261 to Tyr/Ala-269 to Thr) displayed an absorption maximum that was red-shifted by 775 cm^{-1}. Wavelength modulation in the visual pigments responsible for red-green color vision is likely to be governed by retinal-protein interactions involving primarily these two amino acid residues. Furthermore, interactions of hydroxyl-bearing amino acids with the chromophore may be a general mechanism of the opsin shift in visual pigments.

Primate visual pigments share a common chromophore, 11-*cis*-retinal. 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. Seven nonhomologous amino acid substitutions distinguish the human green pigment (λ_{max} = 530 nm) from the human red pigment (λ_{max} = 560 nm) (Table I) (1, 2). Three of these residues were suggested in a genetic analysis of eight primate visual pigments to produce this spectral difference (3). The amino acid at each of these three positions in the rod pigment rhodopsin (λ_{max} = 500 nm) matches that of the green pigment (4, 5). 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 (bathochromic shift) in absorption maximum relative to rhodopsin would indicate potential relevance in red-green spectral tuning. Previous mutagenesis studies have supported a neutral chromophore binding pocket model in rhodopsin (6, 7). In this model, Glu-113 serves as the counterion for the retinylidene Schiff base. Other putative membrane-embedded carboxyls have been proposed to be protonated and therefore neutral (6–9). Specific protein-chromophore interactions and the general mechanism whereby visual pigments tune to specific λ_{max} values have remained unclear.

We prepared a series of seven mutant pigments by introducing hydroxyl-bearing amino acids into bovine rhodopsin. Significant bathochromic spectral shifts were seen in mutants F261Y and A269T.[1] In double mutants, the effects of the mutations on λ_{max} values were additive, largely confirming by direct experimentation the findings of the genetic analysis. The current results are consistent with a role for hydroxyl-bearing amino acids in spectral tuning of the human red and green pigments. Furthermore, the results in which the λ_{max} of rhodopsin is red-shifted by the introduction of hydroxyl groups into the chromophore binding domain implies that this type of protein-chromophore interaction may be a general mechanism to explain the opsin shift in visual pigments.

EXPERIMENTAL PROCEDURES

Materials—Sources of materials used have been previously reported (6, 10). The peptide used to elute pigments from the immunoaffinity resin as described below was synthesized at the Microchemistry Core Facility of the Memorial Sloan-Kettering Cancer Center. Oligonucleotide synthesis was carried out on an Applied Biosystems model 392 synthesizer, and purification of synthetic DNA was carried out essentially as previously described (11, 12).

Construction of Rhodopsin Mutants—Site-directed mutagenesis was performed using restriction fragment replacement "cassette mutagenesis" (13) in a synthetic gene for rhodopsin (11) that had been cloned into the expression vector as previously described (14). Mutant A164S was prepared by replacement of a 35-base pair *Aha*II-*Sfi*I restriction fragment with a synthetic duplex containing the desired codon alteration. Mutants F261Y, A269T, and F261Y/A269T were prepared by replacement of a 103-base pair *Mlu*I-*Apa*I restriction fragment with the appropriate synthetic duplex. Additional double mutants and the triple mutant were prepared by combining *Kpn*I-*Mlu*I fragments of A164S with each of the other mutants. The nucleotide sequence of all cloned synthetic duplexes described was confirmed by the chain terminator method for DNA sequencing on purified plasmid DNA (15).

Expression of Rhodopsin Mutants—The altered genes were expressed in COS-1 cells following transient transfection by a DEAE-dextran procedure as described (16).

Reconstitution and Purification of Mutant Pigments—COS cells expressing the mutant apoproteins were harvested and then incubated in the presence of 11-*cis*-retinal under dim red light to reconstitute pigments as described (6, 16). The purification procedure employed was based on the immunoaffinity procedure of Oprian *et al.* (16) that was modified as described (6, 17).

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[1] Mutants are designated by the native amino acid residue (single-letter code) and its position number followed by the substituted amino acid residue. For example, in mutant A164S, Ala-164 is replaced by Ser.

TABLE I

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

The numbering system shown is from previous reports of the deduced amino acid sequences of bovine rhodopsin (5), human rhodopsin (4), and human cone pigments (2).

Bovine rhodopsin	Human rhodopsin	Human green	Human red
Ala-164	Ala-164	Ala-180[a]	Ser-180
Phe-261	Phe-261	Phe-277	Tyr-277
Ala-269	Ala-269	Ala-285	Thr-285

[a] A genetic polymorphism was reported at this position that could potentially result in Ser at this position as well (2).

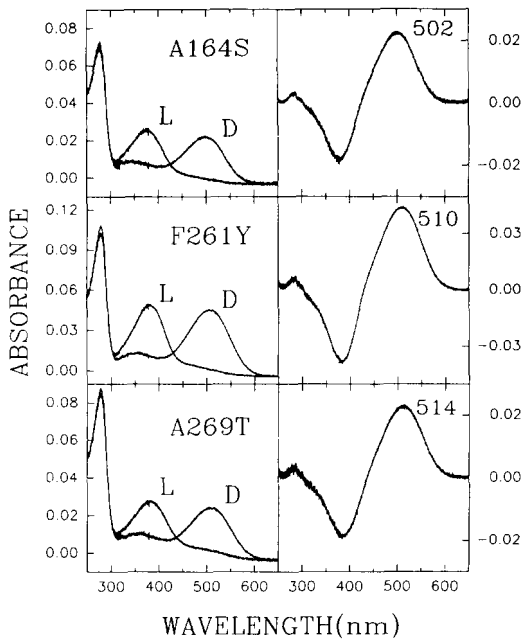

FIG. 1. **UV-visible absorption spectra and photobleaching difference spectra of mutant pigments with single substitutions.** For each pigment, a dark spectrum (*D*) and a spectrum after illumination (*L*) is shown in the *left panel*, and the calculated difference spectrum is shown in the *right panel*. The λ_{max} value determined from the difference spectrum is shown.

Absorption Spectroscopy—Spectroscopy was performed on a λ-19 Perkin-Elmer spectrophotometer. All pigment samples were studied in buffered solutions of 0.1% dodecyl maltoside detergent as previously described (6, 10). Samples were illuminated for 10 s in the cuvette (1.0-cm path length, 20 °C) with a 150-watt light source filtered through a 495-nm long pass filter. Spectra in Figs. 1 and 2 are presented without alteration of data by averaging or smoothing algorithms.

RESULTS AND DISCUSSION

We evaluated seven bovine rhodopsin mutants involving 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) (Fig. 1); three double substitutions; and one triple substitution (Fig. 2). 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 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

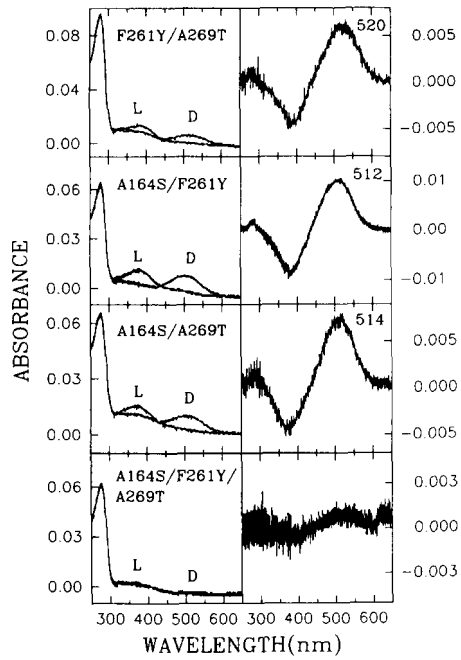

FIG. 2. **UV-visible absorption spectra and photobleaching difference spectra of mutant pigments with multiple substitutions.** For each mutant, a dark spectrum (*D*) and a spectrum after illumination (*L*) is shown in the *left panel*, and the calculated difference spectrum is shown in the *right panel*. The λ_{max} value determined from the difference spectrum is shown. A visible chromophore was not detected for the triple mutant A164S/F261Y/A269T.

TABLE II

Rhodopsin mutants designed to mimic naturally occurring substitutions in green and red pigments

Mutation(s)	λ_{max}[a]	Shift from rho[b]
	nm	*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	ND[c]	

[a] λ_{max} was determined from the peak of photobleaching difference spectra (see Fig. 1). The precision is estimated to be ±2 nm. The λ_{max} of rhodopsin purified from COS cells was 500 nm.

[b] λ_{max} shifts from that of rhodopsin are expressed in wave numbers (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).

[c] Not detected. The triple mutant did not bind 11-*cis*-retinal to form a pigment.

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 II). 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.

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. How-

ever, 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 (8, 9). No significantly red-shifted mutants have been reported other than those involving the Schiff base counterion at position Glu-113 (6, 7, 10, 18).

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 (2). 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 comparisons 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).

Some mutations also affect the efficiency of chromophore binding (Fig. 2). The spectral ratio of absorbance at 280 nm to absorbance at the visible λ_{max} value is a relative measure of the ability of a mutant opsin to bind 11-*cis*-retinal to form a pigment. Assuming that the molar absorptivities of the mutants are the same as that of rhodopsin, the double mutants bind chromophore poorly and the triple mutant not at all. This could result from a direct perturbation of the chromophore binding pocket or from a conformational change that influences, for example, helix-helix interactions. Even mutations in the extracellular domain of rhodopsin have been shown to influence chromophore binding (19). The nature of the mutations, namely the introduction of an hydroxyl group in the cases of Ala to Ser or Phe to Tyr mutations or an hydroxyl plus a methyl in the case of Ala to Thr is not expected to impose major steric constraints. However, the resulting chromophore binding pocket is likely to be more hydrophilic. In this regard, it is noteworthy that 11-*cis*-retinal is not present during the expression of the mutant opsin apoproteins because it is not technically feasible to perform tissue culture in darkness. Perhaps biosynthesis and membrane insertion in the presence of the chromophore would lead to increased pigment formation.

Neitz *et al.* (3) 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 I for a comparison of numbering systems in rhodopsin *versus* cone pigments). 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 9–10-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 substitutions may not be strictly additive. For example, single substitutions in rhodopsin at position 261 (F261Y) and position 269 (A269T) result in red shifts of 400 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 II).

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 than the counterion (6, 7, 9). A neutral chromophore binding pocket model in which dipole and hydrogen bonding interactions predominate has been proposed (6, 7, 20). 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 of mutant rhodopsins and cone pigments that have recently been expressed (21).

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