# Absorption Spectra of the Hybrid Pigments Responsible for Anomalous Color Vision

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**Unequal homologous recombination events between green and red cone pigment genes produce the red-green or green-red hybrid pigment genes found in many individuals with variant color vision. Photobleaching difference absorption spectroscopy of hybrid pigments produced in cultured cells shows that the spectral sensitivity of each hybrid pigment is intermediate between the parental green and red pigment sensitivities. Amino acids encoded by exons 2, 3, 4, and 5 produce spectral shifts at the wavelength of maximal absorbance of 0 to 4, 0 to 4, 3 to 4, and 15 to 21 nanometers, respectively, the exact value depending on the identities of amino acids elsewhere in the hybrid.**

Human color vision is based on absorption of light by three cone pigments that are maximally sensitive at 426 nm (the blue pigment), 530 nm (the green pigment), and either 552 or 557 nm (two polymorphic variants of the red pigment) (*1*, *2*). Among Caucasians, 8% of males and 1% of females differ in their color vision from the majority of the population (*3*). Approximately 30% of males with variant color vision are dichromats, lacking either red pigment sensitivity [protanopes ($G^+R^-$)] or green pigment sensitivity [deuteranopes ($G^-R^+$)]. The remaining 70% are anomalous trichromats, either protanomalous ($G^+R'$) or deuteranomalous ($G'R^+$). Psychophysical experiments show that anomalous trichromats possess pigments with spectral sensitivities that are between the normal red and green sensitivities (*4*), whereas dichromats are missing one of the three pigments (*5*).

Dichromacy and anomalous trichromacy are caused by unequal homologous recombination within the tandem array of genes that encode the red and green visual pigments (*6*–*8*). In $G^+R^-$ and $G^+R'$ subjects, a 5′red–3′green hybrid gene is found in place of the normal red pigment gene, whereas $G'R^+$ subjects have one or more 5′green–3′red hybrid genes. $G^-R^+$ subjects either have lost all green pigment genes or have replaced the remaining green pigment gene with a 5′green–3′red hybrid gene. The hybrid genes are predicted to encode the variant pigments that underlie dichromacy and anomalous trichromacy.

Coding differences between the red and green pigment genes are confined to exons 2 to 5 (*9*), and current evidence indicates that the most common hybrids in the human population correspond to homologous recombination events in the intervals between these exons (*7*, *10*, *11*). Nine rather than six hybrids are expected because of the existence of two variants of the red pigment that differ by the presence of Ala or Ser at position 180 encoded by exon 3 (*1*, *12*). Each hybrid pigment is referred to here by an abbreviation that reflects the origin of the exons that encode it. For example, R3G4(Ala$^{180}$) is a hybrid pigment encoded by a gene in which exons 1 to 3 are derived from a red pigment gene encoding Ala at position 180 in exon 3 and in which exons 4 to 6 are derived from a green pigment gene.

To define the spectral sensitivities of the nine most common hybrid pigments, we expressed cDNAs encoding the corresponding hybrid apoproteins (*13*) by transient transfection of a human embryonic kidney cell line (*14*). Photobleaching difference absorption spectra showed that each hybrid apoprotein produces a photolabile visual pigment (*15*) (Fig. 1 and Table 1). The wavelengths of maximal absorption ($\lambda_{max}$) and half-maximal absorption along the long wavelength limb ($\lambda_{1/2max}$) of each hybrid pigment fall within the interval defined by the absorption spectra of the green pigment and the corresponding allelic variant of the red pigment (*16*). Comparison of the hybrid and wild-type pigment spectra reveals that sequence differences encoded by exon 5 are the primary determinant of red, green, and hybrid pigment spectral sensitivity. Swapping these sequences produces spectral shifts of 15 to 20 nm in $\lambda_{max}$ and of 24 to 26 nm in $\lambda_{1/2max}$ [that is, green as compared to (versus) G4R5, red(Ala$^{180}$)

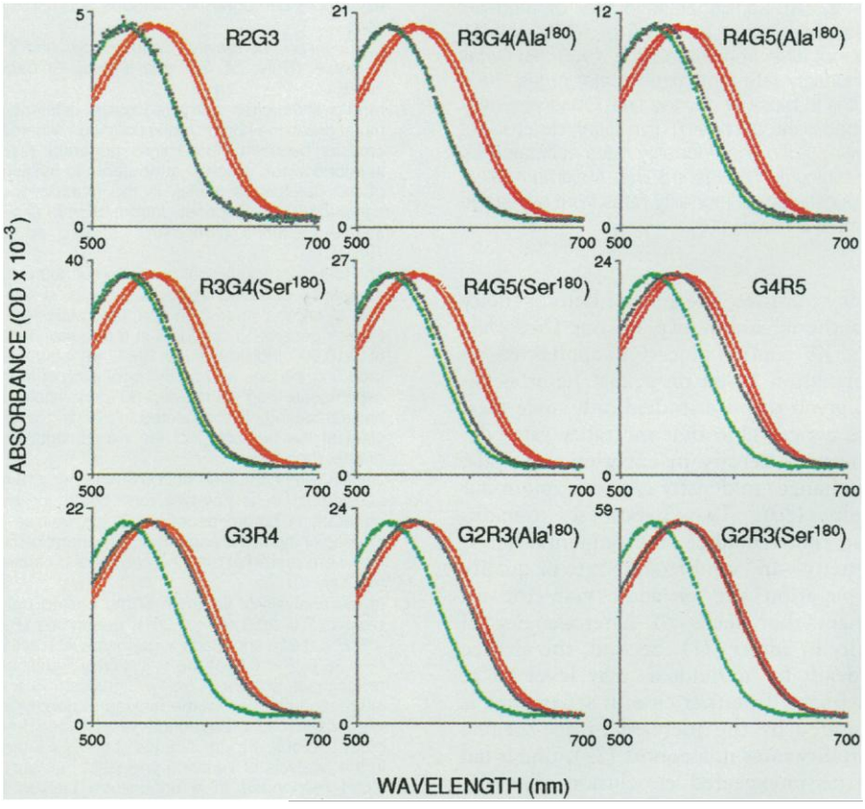


**Fig. 1.** Photobleaching difference absorption spectra of the nine human hybrid pigments (in black) superimposed on photobleaching difference absorption spectra of the green pigment (in green) and the two variants of the red pigment (in red), red(Ala$^{180}$) (left) and red(Ser$^{180}$) (right) (*1*). Absorbance values refer to the hybrid pigment spectra. The two red pigment spectra and the green pigment spectrum (a summed average of two spectra) were scaled to match the absorbance values of each hybrid pigment at 700 nm and at $\lambda_{max}$. For each hybrid pigment, the spectrum from a single experiment is shown.

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versus R4G5(Ala$^{180}$), and red(Ser$^{180}$) versus R4G5(Ser$^{180}$)] (*17*).

In the 5′red–3′green hybrid series, red pigment sequences encoded by exons 2 and 3 (the latter containing Ala$^{180}$) produce little change in $\lambda_{max}$, although there is a 2- to 4-nm red shift (that is, toward longer wavelengths) in $\lambda_{1/2max}$ [green versus R2G3 and R3G4(Ala$^{180}$)]. These results are consistent with those obtained previously by electroretinography of a subject with a single R3G4(Ala$^{180}$) hybrid gene (*10*). Red pigment sequences encoded by exon 4 induce a further red shift of 3 nm and 2 to 4 nm in the $\lambda_{max}$ and $\lambda_{1/2max}$, respectively, of the hybrids [R3G4(Ala$^{180}$) versus R4G5(Ala$_{180}$) and R3G4(Ser$^{180}$) versus R4G5(Ser$^{180}$)]. In the four 5′red–3′green hybrids with red pigment sequences encoded by exon 3, substitution of Ala$^{180}$ for Ser$^{180}$ produces a 4-nm red shift in $\lambda_{max}$ and a 2- to 4-nm red shift in $\lambda_{1/2max}$.

A somewhat larger effect of sequence differences encoded by exons 2 through 4 is seen in the 5′green–3′red hybrid series. For hybrids that contain either Ser$^{180}$ or Ala$^{180}$, replacement of red pigment sequences encoded by exon 2 with the corresponding green pigment sequences leads to 3- to 4-nm and 3-nm blue shifts (that is, toward shorter wavelengths) in $\lambda_{max}$ and $\lambda_{1/2max}$, respectively [red(Ala$^{180}$) versus G2R3(Ala$^{180}$) and red(Ser$^{180}$) versus G2R3(Ser$^{180}$)]. Green pigment sequences encoded by exon 3, which contains Ala at position 180, are indistinguishable in their spectral effects from the corresponding red(Ala$^{180}$) pigment sequences [G2R3(Ala$^{180}$) versus G3R4], which indicates that the polymorphic Leu/Met at position 153 (*9*) in exon 3 does not affect spectral tuning. Green pigment sequences encoded by exon 4 induce a blue shift in both $\lambda_{max}$ and $\lambda_{1/2max}$ of 4 nm (G3R4 versus G4R5). In the two hybrids with red pigment sequences encoded by exon 3, G2R3(Ser$^{180}$) and G2R3(Ala$^{180}$), replacement of Ala$^{180}$ by Ser$^{180}$ produces $\lambda_{max}$ and $\lambda_{1/2max}$ red shifts of 3 and 5 nm, respectively.

The hybrid pigment absorption spectra are consistent with models of spectral tuning based on a comparison of amino acid sequences and spectral sensitivities of primate visual pigments (*18*, *19*) and with the effects of amino acid replacements in bovine rhodopsin (*20*). We observe that sequence differences encoded by exon 5 are responsible for a 15- to 21-nm shift in $\lambda_{max}$, consistent with the proposal that substitution of Tyr$^{277}$ or Thr$^{285}$ (residues in the red pigment encoded by exon 5) for Phe or Ala (the corresponding residues in the green pigment) produces red shifts of 9 and 15 nm, respectively (*18*). The 3- to 4-nm effect of sequence differences encoded by exon 4 may account for the decreased red pigment sensitivity in males who carry Thr, Ser, and Val, residues encoded by exon 4 of the green pigment gene, in place of Ile$^{230}$, Ala$^{233}$, and Met$^{236}$, respectively, encoded by the red pigment gene (*12*).

The absorption spectra of the hybrid pigments, together with those of the green and red pigments reported earlier (*1*, *2*), provide a framework for understanding variations in red-green color vision. If the 5′red–3′green hybrid gene present in a $G^+R^-$ or $G^+R'$ subject is R4G5(Ser$^{180}$) ($\lambda_{max}$ = 536 nm), red-green color vision will be based on the 6-nm difference between the absorption maximum of this pigment and that of the normal green pigment ($\lambda_{max}$ = 530 nm). If the hybrid is either R3G4(Ser$^{180}$) or R4G5(Ala$^{180}$), red-green color vision will be based on an absorption difference of only a few nanometers, and if the hybrid is either R2G3 or R3G4(Ala$^{180}$), the absorption spectrum will be nearly identical to that of the green pigment. In the last case, chromatic discrimination based on the difference between the hybrid and a normal green pigment would be unlikely.

In subjects with 5′green–3′red hybrid pigments, the phenotype depends on both the point of crossover in the hybrid and whether the accompanying red pigment contains Ala$^{180}$ or Ser$^{180}$. In subjects with a G4R5 pigment ($\lambda_{max}$ = 545 nm) and a red(Ser$^{180}$) pigment ($\lambda_{max}$ = 557 nm), chromatic discrimination would be based on a spectral separation of 12 nm. Hybrids with absorption spectra that are further red-shifted—for example, G3R4 ($\lambda_{max}$ = 549 nm)—would give smaller chromatic signals, especially in combination with red(Ala$^{180}$) ($\lambda_{max}$ = 552 nm). In the extreme case, a G1R2 hybrid would correspond to the pigment encoded by one of the two normal red alleles. If the accompanying normal red pigment gene encoded an identical pigment, the phenotype would be $G^-R^+$ dichromacy, whereas an Ala/Ser$^{180}$ difference between the two would lead to $G'R^+$ anomalous trichromacy (*21*).

**Table 1.** Quantitative analysis of red-green and green-red hybrid pigment spectra. For each photobleaching difference absorption spectrum (*15*), two best-fitting fifth-order polynomials were calculated, one for the 100-nm region centered about the approximate absorption maximum and another for the 120-nm region centered 10 nm toward the long wavelength side of the absorption maximum. The wavelengths of maximal absorption ($\lambda_{max}$) and half-maximal absorption along the long wavelength limb ($\lambda_{1/2max}$) were measured from the first and second fifth-order curves, respectively. Included for comparison are the $\lambda_{max}$ and $\lambda_{1/2max}$ for the parental green and red pigments (*1*). Each experiment represents an independent transfection. SEM, standard error of the mean; SD, standard deviation; OD, optical density.

| Pigment | Mean $\lambda_{max}$ (nm) | SEM (nm) | SD (nm) | Mean $\lambda_{1/2max}$ (nm) | SEM (nm) | SD (nm) | Experiments (*n*) | Mean amplitude (OD × $10^{-3}$) |
|---|---|---|---|---|---|---|---|---|
| Green | 529.7 | | | 576.0 | | | | |
| R2G3 | 529.5 | 0.8 | 2.6 | 577.7 | 0.4 | 1.3 | 10 | 4 |
| R3G4(Ala$^{180}$) | 529.0 | 0.4 | 1.0 | 579.6 | 0.2 | 0.5 | 5 | 10 |
| R3G4(Ser$^{180}$) | 533.3 | 0.5 | 1.0 | 581.3 | 0.5 | 1.0 | 4 | 14 |
| R4G5(Ala$^{180}$) | 531.6 | 0.6 | 1.8 | 581.4 | 0.2 | 0.5 | 8 | 7 |
| R4G5(Ser$^{180}$) | 536.0 | 0.7 | 1.4 | 585.5 | 0.5 | 1.0 | 4 | 16 |
| Red(Ala$^{180}$) | 552.4 | | | 607.3 | | | | |
| Red(Ser$^{180}$) | 556.7 | | | 612.1 | | | | |
| G2R3(Ala$^{180}$) | 549.6 | 0.4 | 0.9 | 604.4 | 0.2 | 0.5 | 5 | 32 |
| G2R3(Ser$^{180}$) | 553.0 | 0.7 | 1.4 | 609.0 | 0.4 | 0.8 | 4 | 39 |
| G3R4 | 548.8 | 0.6 | 1.3 | 604.5 | 0.3 | 0.6 | 4 | 11 |
| G4R5 | 544.8 | 0.8 | 1.8 | 600.6 | 0.5 | 1.1 | 5 | 13 |

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9. Fifteen amino acids differ between the human red(Ser$^{180}$) and green pigments identified in cloned genomic DNA. The cDNA clones for the red(Ala$^{180}$) and green pigments (hs7 and hs2, respectively) (*8*) encode proteins that differ by 16 amino acids. The additional difference is due to a Thr at position 103 in the green pigment cDNA clone hs2 versus a Ser at this position in the red and green pigment genomic clones gJHN33 and gJHN21 and the red pigment cDNA clones hs4 and hs7 (*8*). A Leu/Met difference at position 153 between red and green pigment genomic clones is reversed in the cDNA clones (*8*).
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13. Hybrid pigment genes were prepared as follows. Red or green pigment cDNA clones (hs7 or hs2, respectively, the latter reconstructed at its 5′ end to include the first 14 codons missing from the original cDNA clone) were inserted into the expression vector pCIS (*14*) as described (*1*), linearized at a restriction site adjacent to the cDNA insert, digested for a variable length of time with exonuclease III (Promega, Madison, WI), and then further digested with nuclease S1 (Promega). The partially digested red or green pigment cDNA fragment was used to prime synthesis on a single-stranded template that contained either the green or red pigment cDNA, respectively. After transfection of an *Escherichia coli* mutant deficient in mismatch repair (*mut L*), appropriate hybrids were identified by oligonucleotide hybridization and DNA sequencing. For those hybrids that contained a red pigment–derived exon 3, hybrids that contained a Ser at position 180 were constructed from the Ala-containing hybrids by oligonucleotide-directed site-specific mutagenesis. The 3′ untranslated region of each 5′red–3′green hybrid cDNA was replaced with the 3′ untranslated region of the red pigment cDNA hs7. The entire nucleotide sequence of each hybrid was determined on one strand both to confirm the identity of the hybrid and to rule out spurious mutations.
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15. The hybrid pigment cDNAs were used to transiently transfect 30 to 50 10-cm plates of a human embryonic kidney cell line (293S; American Type Culture Collection CRL 1573). Twenty-four hours after transfection, cells were harvested, and cell membranes that contained visual pigment apoprotein were isolated (*1*). After incubation with 11-*cis*-retinal, the membranes were solubilized in 2% CHAPS, and the soluble fraction was used for photobleaching difference absorption spectroscopy (*1*). Differences in yield of as much as tenfold were observed for different hybrid pigments (Table 1). Pigment yields after transient transfection were: green < 5′red–3′green ≤ 5′green–3′red < red (Table 1) (S. L. Merbs and J. Nathans, unpublished results). In general, the vast majority of the cone pigment apoprotein exists in an aggregated form as judged by protein immunoblot (S. L. Merbs and J. Nathans, unpublished results), which suggests that cone pigment apoproteins are unstable in tissue culture cells.
16. The most reproducible portion of the absorption spectrum is the long wavelength limb because of lower light scattering at long wavelengths and because some of the hybrid pigment curves show a distortion of their short wavelength limbs, centered at approximately 440 nm. The presence and size of this distortion are variable and increase with longer bleaching times, with an amplitude between 0 and 25% that of the visual pigment peak. It appears to have little or no effect on the long wavelength limbs of the hybrid pigment spectra. This distortion most likely represents formation of other photolabile Schiff's bases of 11-*cis*-retinal.
17. When measured on a wavelength scale, the slope of the long wavelength limb of the green pigment is steeper than that of either of the two red pigments. The values for $\lambda_{max}$ of green and red-(Ala$^{180}$) or red(Ser$^{180}$) pigments differ by 22 or 27 nm, respectively, whereas their values for $\lambda_{1/2max}$ differ by 31 or 36 nm, respectively (*1*). This discrepancy between $\lambda_{max}$ shifts and $\lambda_{1/2max}$ shifts can also be seen in a comparison of G4R5 and the green pigment, R4G5(Ala$^{180}$) and the red(Ala$^{180}$) pigment, or R4G5(Ser$^{180}$) and the red(Ser$^{180}$) pigment. This difference in slope of the long wavelength limbs is still evident when the logarithm of the pigment spectral sensitivity is plotted versus the logarithm of the pigment spectra wavenumber [D. A. Baylor, B. J. Nunn, J. L. Schnapf, *J. Physiol.* **390**, 145 (1987)].
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22. We thank C. Riley, A. Collector, and C. Waldron for synthesis of oligonucleotides; C. Gorman, A. Levinson, and R. Kline (Genentech) for making available the pCIS vector and 293S cell line; L. Stryer (Stanford University) and the Hoffman–La Roche Co. for gifts of 11-*cis*-retinal; and I. Chiu, J. Macke, and C. Weitz for comments on the manuscript. S.L.M. is a trainee of the Medical Scientist Training Program (NIH). Supported by the Howard Hughes Medical Institute and the National Eye Institute (NIH).

8 June 1992; accepted 4 August 1992

# Isomerase and Chaperone Activity of Prolyl Isomerase in the Folding of Carbonic Anhydrase

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**Several proteins have been discovered that either catalyze slow protein-folding reactions or assist folding in the cell. Prolyl isomerase, which has been shown to accelerate rate-limiting *cis-trans* peptidyl-proline isomerization steps in the folding pathway, can also participate in the protein-folding process as a chaperone. This function is exerted on an early folding intermediate of carbonic anhydrase, which is thereby prevented from aggregating, whereas the isomerase activity is performed later in the folding process.**

For many proteins, in vitro protein folding is a spontaneous process that does not require input of energy or any assisting factors (*1*). Folding in vivo, however, seems for many proteins to be mediated by several protein factors. These helper proteins are divided into two classes: (i) folding catalysts, such as the enzymes protein disulfide isomerase and prolyl isomerase (PPIase) and (ii) molecular chaperones that bind transiently to folding intermediates and thereby prevent incorrect interactions leading to aggregation (*2–5*). We have noted that with the use of PPIase in the study of the rate-limiting step of the refolding of human carbonic anhydrase II (HCA II), which contains 15 *trans*- and 2 *cis*-prolines (residues 30 and 202) (*6*), PPIase has a positive effect on both the kinetics and the yield of active HCA II (*7*). Because aggregation has been shown to lead to incomplete recovery of native HCA II in unassisted refolding of denatured enzyme (*8*), two questions arise. First, is the higher HCA II yield obtained in the presence of PPIase the result of a reduction of the time of exposure of hydrophobic surfaces in folding intermediates that is caused by catalysis of slow *cis-trans* isomerization reactions? That is, does PPIase facilitate the folding process in the way that is generally believed (*9*), or alternatively, does PPIase act as a chaperone by binding to an early folding intermediate, which is thereby protected from unproductive interactions that otherwise lead to aggregation?

PPIase accelerates the reactivation of denatured HCA II [1-hour incubation in 5 M guanidine hydrochloride (GuHCl)] and also increases the recovery of active enzyme: with PPIase, the half-time ($t_{1/2}$) of reactivation is lowered from 9 to 4 min, and the yield of enzyme is increased from 70 to 100% (Fig. 1). These stimulating effects are completely abolished when the PPIase inhibitor cyclosporin A (CsA) is added at the onset of refolding. No effect on the yield or the kinetics of the reactivation was noted when bovine serum albumin was added to the same concentration as that of PPIase, which rules out the possibility that these effects are caused by general protein interactions.

If HCA II is allowed to be in its denatured state (in 5 M GuHCl) for only 10 s, *cis-trans* isomerization of peptidyl-proline bonds is greatly limited ($t_{1/2}$ = ~2 min). However, within this 10-s incubation, full denaturation is achieved, as judged by activity and ultraviolet absorbance (absorbance = 292 nm) measurements. The reactivation of this short-time (10-s) denatured HCA II proceeds much faster ($t_{1/2}$ =

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