Spectral Tuning in the Human Blue Cone Pigment†

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ABSTRACT: The first determination of the absolute absorption maximum of the human blue cone visual pigment is presented. After expression in COS cells, reconstitution with 11-*cis*-retinal, and purification, the blue pigment exhibits an absolute absorption maximum of 414 nm. The pigment reacts rapidly with hydroxylamine in the dark and is capable of activating bovine rod transducin in a light-dependent manner. Products of mutations of proposed spectral tuning residues in the blue pigment do not behave as predicted when using rhodopsin mutants as a model. Mutations of amino acids in the ring portion of the chromophore binding pocket of rhodopsin serve well as a predictive model for mutations in the blue pigment, but mutations near the Schiff base do not.

Human color vision is mediated by three cone photoreceptor classes containing either a blue, green, or red visual pigment. Of these, the blue pigment has been the least studied, and there is considerable uncertainty with regard to its absorption maximum, with a range in reported values from 419 to >440 nm (*1*−*4*). As interest in the blue pigment increases, largely as a consequence of the recent cloning of the related ultraviolet-sensitive pigments (*5*, *6*), so does the need for reliable characterization of the blue pigment. Certainly, any study of the underlying mechanisms of spectral tuning of this pigment must begin with an accurate determination of the absolute absorption maximum. We report here the first determination of the absolute absorption maximum of the human blue pigment, 414 nm. In addition, we show that products of mutations of proposed spectral tuning residues in the blue pigment do not behave as predicted when using rhodopsin mutants as a model.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of the Rhodopsin and Blue Cone Opsin Genes. The synthetic human blue cone opsin and rhodopsin genes used in this study were designed to contain unique restriction sites to facilitate cassette mutagenesis (*3*, *7*). Details concerning the construction and characterization of the genes can be found in previous publications (*3*, *7*). All procedures for DNA manipulation, mutation of the opsin genes, DNA sequence analysis, and transient expression of the opsin genes in COS cells were performed as previously described (*7*−*9*).

Reconstitution and Purification of the Proteins. Transfected COS-1 cells were harvested 72 h after initial exposure to DEAE-dextran and DNA. Procedures for reconstitution of the pigments with 11-*cis*- and 9-*cis*-retinal [11-*cis*-retinal was generously provided by R. Crouch and NEI; 9-*cis*-retinal was from Sigma (St. Louis, MO)], solubilization of the COS cell membranes with 1% DM,[1] and purification of the proteins by immunoaffinity chromatography on the 1D4-Sepharose 4B matrix have been described previously (*3*). Pigments were eluted from the immunoaffinity column in 0.01% DM and concentrated approximately 10-fold using Centricon-30 concentrators (Amicon, Inc., Beverly, MA) for the spectral studies. We estimate that the final concentration of DM was approximately 0.1%.

Absorption Spectroscopy. UV−visible absorption spectra were recorded using a Hitachi model U-3210 spectrophotometer that was specifically modified by the manufacturer for use in a dark room. Data were acquired with the aid of a Gateway 2000 4DX2-50V microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). All spectra were recorded on samples with a 1.0 cm path length in thermostated cell holders with the temperature maintained at 4 °C. Difference spectra were generated after correction of the bleached spectrum for a 5% dilution resulting from addition of hydroxylamine. The λ_{max} values reported in Figures 1, 2, and 5−7 were determined from the first derivative of a fourth-order polynomial fit to a 40 nm region surrounding each maximum.

Determination of the Extinction Coefficient. The extinction coefficient for the blue pigment was determined by acid trapping of the chromophore as follows. After the dark-state spectrum had been recorded, 2 μL of 12 N HCl was added to the sample to denature the protein and trap the chromophore as a protonated Schiff base with a characteristic absorption maximum at 440 nm. Since acid trapping removes the influence of the protein from the chromophore, the human blue pigment and bovine rhodopsin should give spectrally identical species upon acid denaturation. Therefore, the known extinction coefficient for rhodopsin at 500 nm (*10*)

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[1] Abbreviations: DM, *n*-dodecyl β-D-maltoside; λ_{max}, absorption maximum (maxima); UV, ultraviolet; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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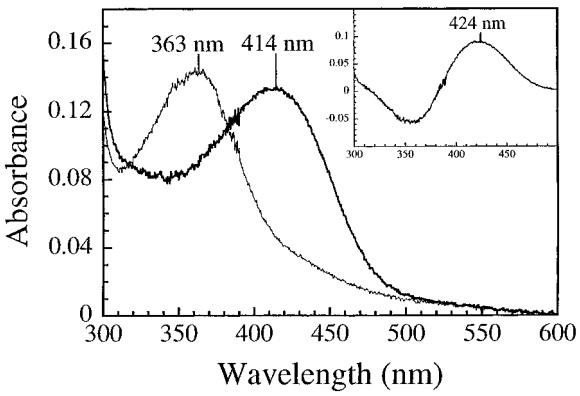

FIGURE 1: Absolute absorption spectrum of the human blue cone pigment. After the absorption spectrum was recorded, the sample was exposed to light in the presence of 50 mM hydroxylamine (pH 7.0) and the spectrum of the resulting retinal oxime was recorded again (λ_{max} = 363 nm). The inset shows the difference spectrum that was obtained by subtracting the absolute spectrum of the retinal oxime from that of the dark sample.

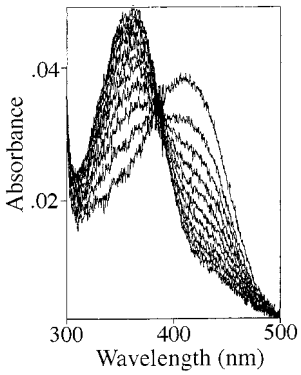

FIGURE 2: Reaction of the wild-type blue cone pigment with hydroxylamine. Spectra were recorded at 7 min intervals after the addition of 50 mM hydroxylamine (from a neutralized stock solution) to the purified pigment in 50 mM HEPES (pH 6.5) containing 140 mM NaCl and 0.1% (w/v) DM in the dark. The temperature was maintained at 4 °C. In order of decreasing absorbance at 414 nm (increasing absorbance at 363 nm corresponding to retinal oxime), spectra were recorded 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 min after the addition of hydroxylamine.

may be used to determine an extinction coefficient for the human blue pigment at 414 nm through the intermediacy of the acid-trapped species. That is, we used the extinction coefficient for native rhodopsin at 500 nm to determine the extinction coefficient of acid-trapped rhodopsin at 440 nm. We used the extinction coefficient for the acid-trapped species to determine the concentration of the blue pigment from its acid-trapped spectrum, and we used the concentration of the blue pigment to determine an extinction coefficient at 414 nm for the native blue pigment.

Assay for Activation of Transducin. Light-dependent activation of bovine rod cell transducin was assessed by following the binding of [^{35}S]GTPγS as described previously (*9*).

Correction of the Absorbance Spectrum of the Ser87Gly Blue Pigment Mutant. The absolute absorption spectrum of the Ser87Gly mutant was broader than the spectrum for the wild-type pigment as a result of a contribution from additional absorbance on the short-wavelength end of the spectrum (Figure 5). The source of the short-wavelength absorbance is not known, but it likely arises from either light scattering or possibly free retinal released as a result of the thermal instability of this mutant. Because it was crucial to determine the absolute maximum for the Ser87Gly mutant without interference from any possible nonpigment contributions, the spectrum was corrected as follows. After the dark-state spectrum had been recorded, 2 μL of 12 N HCl was added to the sample to denature the protein and trap the chromophore as a protonated Schiff base with a characteristic absorption maximum at 440 nm (Figure 7A). The concentration of the original mutant pigment was then determined from the acid-trapped spectrum at both 440 and 470 nm using extinction coefficients determined as described above. A difference spectrum was then generated by subtracting the acid-trapped spectrum from that of the dark state (Figure 7C). Sources of nonpigment absorbance such as light scattering or free retinal are not affected by acid and, therefore, are eliminated by subtraction in the difference spectrum. An absorption spectrum for the acid-trapped chromophore from the wild-type blue pigment (Figure 7B) scaled to the same concentration was then added to the difference spectrum of the Ser87Gly mutant (Figure 7C) to generate the final corrected absolute spectrum for the mutant (Figure 7D).

RESULTS AND DISCUSSION

The absolute absorption spectrum of the human blue cone pigment purified by immunoaffinity chromatography from COS cells transfected with a gene for the blue opsin exhibits a maximum at 414 nm, as shown in Figure 1. The extinction coefficient of the blue pigment at 414 nm is 51 000 M^{-1} cm^{-1}, a value greater than that reported for rhodopsin (42 700 M^{-1} cm^{-1} at a λ_{max} of 500 nm) (*10*). The 414 nm maximum is significantly blue-shifted with respect to most previous estimates for the maximum of the blue pigment but is closest to the value of 419.0 ± 3.6 nm determined by Dartnall et al. (*2*) using microspectrophotometry of individual human cone cells. It is also very close to the 415 nm maximum reported by Yoshizawa and co-workers for the evolutionarily related chicken violet pigment (*11*). The difference spectrum for the human blue pigment, shown in the inset of Figure 1, exhibits a maximum at 424 nm, in good agreement with the previously reported value (*3*). Note that the maximum from the difference spectrum (424 nm) is red-shifted relative to the absolute maximum (414 nm) because of overlap of the blue pigment spectrum with that of the retinal oxime product formed upon photobleaching in the presence of hydroxylamine.

In experiments designed to further characterize the blue pigment biochemically, (1) the protein was found to not be able to generate a pigment with 9-*cis*-retinal (not shown) in contrast to the known ability of rod opsin to form isorhodopsin (λ_{max} = 485 nm) with 9-*cis*-retinal; (2) the blue pigment was found to react with 50 mM hydroxylamine in the dark to give retinal oxime and blue opsin with a pseudo-first-order rate constant of about 2 h^{-1} at 4 °C and pH 6.5, as shown in Figure 2; and (3) the blue pigment was found to be capable of activating bovine rod cell transducin in a light-dependent manner, as shown in Figure 3, although with a specific activity on the order of 50−75-fold lower than that of rhodopsin (106 pmol min^{-1} $pmol^{-1}$).

On the basis of modeling studies and sequence alignments among short-wavelength pigments, Lin et al. (*12*) identified

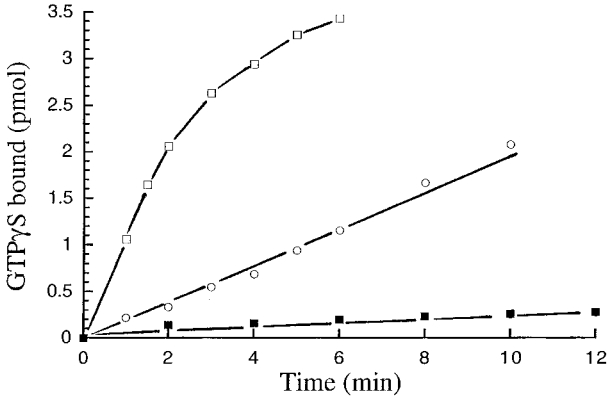

FIGURE 3: Light-dependent activation of bovine transducin by the wild-type blue cone pigment. Transducin activity was assayed by measuring the level of binding of [^{35}S]GTPγS (*22*) using 15 nM blue cone pigment purified from transfected COS cells. Bovine rhodopsin at a concentration of 1 nM (also purified from transfected COS cells) was assayed as a control: (black symbols) reaction carried out in the dark, (white symbols) reaction after the mixture was exposured to light, (○) blue pigment, and (□ and ■) rhodopsin.

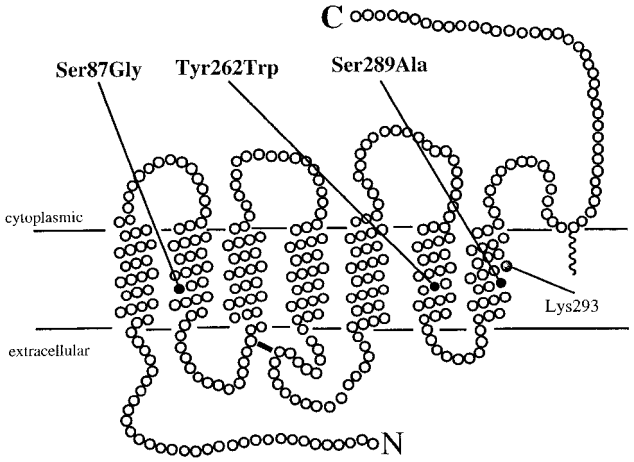

FIGURE 4: Schematic diagram for the human blue cone visual pigment. The three amino acids selected for mutagenesis, Ser87, Tyr262, and Ser289, are highlighted. In each case, the amino acid in the blue pigment is followed by the position number in the blue sequence, followed by the amino acid at that position in bovine rhodopsin. Lys293 is the site of chromophore attachment.

a number of amino acid residues in rhodopsin and the human blue cone pigment that might be important for regulating the spectral characteristics of these two proteins. When these amino acids were changed in rhodopsin to the corresponding residues in the blue pigment, three of the substitutions resulted in large blue shifts in the absorption spectrum, suggesting that these residues are important for spectral tuning in rhodopsin and that they may also be important in positioning the short-wavelength maximum of the human blue pigment. The amino acids in rhodopsin are Gly90, Trp265, and Ala292, which correspond to Ser87, Tyr262, and Ser289 in the human blue pigment (see Figure 4). As shown by Lin et al. (*12*), changing Gly90 to Ser results in an 11 nm blue shift in the absorption maximum of rhodopsin, Trp265 to Tyr a 15 nm blue shift, and Ala292 to Ser a 10 nm blue shift. If these three residues are important for spectral tuning in the blue pigment, and if rhodopsin is an appropriate model for the blue pigment in spectral tuning studies, then changing each amino acid in the blue pigment to the corresponding residue in rhodopsin should result in a

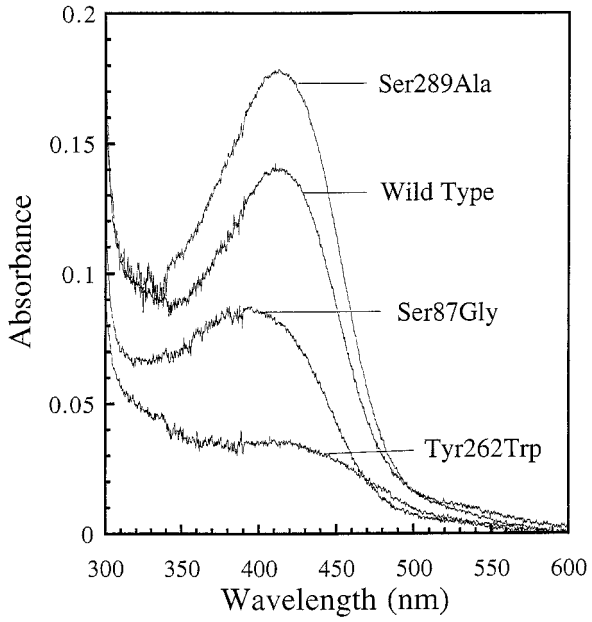

FIGURE 5: Absolute absorption spectra for the wild type and the three blue pigment mutants: Ser87Gly, Tyr262Trp, and Ser289Ala.

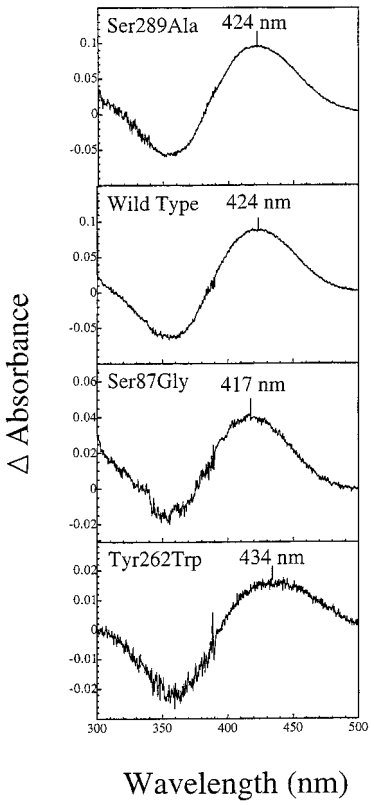

FIGURE 6: Difference spectra for the wild type and the three blue pigment mutants: Ser87Gly, Tyr262Trp, and Ser289Ala. Difference spectra were obtained as described in the legend of Figure 1.

significant red shift in the absorption spectrum of the blue pigment. In accord with this expectation, changing Tyr262 in the blue pigment to Trp did in fact result in a significant red shift (Figure 5), and although the low pigment yield for this mutant made a determination of the absolute absorption maximum difficult, the difference maximum (where distortion from light scattering has been eliminated) is clearly seen to shift from 424 nm in the wild type to 434 nm in the mutant (Figure 6). However, in stark contrast to the mutation of

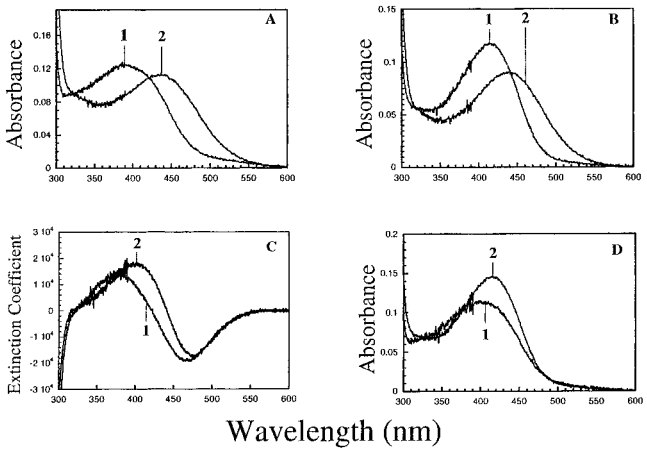

FIGURE 7: Corrected absorption spectrum of the Ser87Gly mutant blue pigment. (A) Dark-state (1) and acid-trapped (2) spectra of the Ser87Gly mutant blue pigment. (B) Dark-state (1) and acid-trapped (2) spectra of the wild-type blue pigment. (C) Difference spectra generated by subtracting the acid-trapped spectrum from the dark-state spectrum for the Ser87Gly mutant (1) and the wild-type blue pigment (2). (D) Corrected spectrum (see Experimental Procedures) for the Ser87Gly mutant (1). The spectrum for the wild-type pigment (2) is shown for comparison.

Tyr262, changing Ser289 to Ala had no effect on the spectral properties of the pigment, and changing Ser87 to Gly caused a significant blue shift, a direction which is the opposite of that predicted from mutation of this residue in rhodopsin (Figures 5 and 6). Although it appears that free retinal or light scattering may have contributed slightly to the blue shift of the Ser87Gly mutant blue pigment spectrum shown in Figure 5, the corrected spectrum shown in Figure 7D for the Ser87Gly mutant had a λ_{max} of 404 nm, resulting in a net blue shift of 10 nm relative to that of the wild-type blue pigment. Thus, rhodopsin appears to serve well as a model for the blue pigment at position 262, but poorly at positions 87 and 289.

Photochemical cross-linking studies in rhodopsin have shown convincingly that Trp265 (Tyr262 in blue) is located close to the β-ionone ring of the 11-*cis*-retinal chromophore (*13*, *14*), whereas mutagenesis studies indicate that Gly90 (Ser87) (*15*) and Ala292 (Ser289) (*16*−*18*) are located near the Schiff base nitrogen. The serine substitutions at positions 90 and 292 in rhodopsin are thought to blue shift the λ_{max} as a result of interactions between the polar hydroxyl group of Ser and the positive charge on the Schiff base nitrogen (*12*, *17*, *18*). We conclude that mutation of an amino acid in the ring portion of the chromophore binding pocket of rhodopsin serves well as a predictive model for mutations in the blue pigment, but mutations near the Schiff base do not. It appears from these results that rhodopsin and the blue pigment differ fundamentally in the Schiff base region of the chromophore, and it is possible that this difference arises because the blue pigment chromophore has an unprotonated Schiff base. The absence of a shift in the maximum for the Ser292 to Ala mutation in the blue pigment and the blue shift observed for the Ser89 to Gly mutation are both consistent with this suggestion. An unprotonated Schiff base would be unexpected as every vertebrate visual pigment identified to date contains in its sequence a highly conserved glutamate residue corresponding to the Schiff base counterion, Glu113, in rhodopsin (*19*−*21*). An obvious experiment suggested by this discussion is mutation of this residue (Glu110Gln) in the human blue pigment. Unfortunately, when we introduced this mutation in the blue opsin, the mutant failed to bind 11-*cis*-retinal. Thus, although the mutagenesis results presented here are consistent with an unprotonated Schiff base in the human blue pigment, further studies will be needed to unequivocally establish the protonation state of the Schiff base.

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