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Spectra of human L cones

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Abstract

Variations in the amino acid sequences of the human cone opsins give rise to spectrally variant subtypes of L and M cone pigments even in the population with normal color vision. In vitro mutagenesis studies have shown that a limited number of amino acid substitutions produce shifts in the wavelength sensitivity. Presented here are results comparing electrophysiological measurements of single human cones with the expressed cone pigment gene sequences from the same retina. In a sample of eight long-wavelength sensitive cone (L cone) spectra obtained from five donors the precise spectral sensitivities, measured in situ, of the two most commonly occurring spectral variants were determined. The peak sensitivity of the L_{ser180} cone was 563 nm while that of the L_{ala180} cone was 559 nm.

Keywords: Action spectrum; Human cone pigments; Polymorphism; Electrophysiology; Colour vision

1. Introduction

Psychophysical differences in visual sensitivity among men with color vision defects are attributed to variations in spectral absorption of the cone pigments [1,2]. Alpern and Wake [3] suggested that the normal pigments must vary as well. By examining the genes encoding different spectral variants of L and M pigments, Neitz and co-workers [4] identified three amino acid positions of the cone pigment apoproteins (opsins) which were principally responsible for tuning the spectra of the pigments. Two of the amino acid positions, 277 and 285 together are responsible for a large spectral difference (>16 nm); the amino acid at position 180, produces a smaller shift (4–7 nm) and was identified as the candidate for producing variation in color matching among individuals with normal color vision. Studies in which pigment genes and color vision behavior were characterized in the same individuals [5–7] confirmed that the presence of serine 180 (versus alanine 180) correlated strongly with higher visual sensitivity to red light.

The identities of the amino acid positions among the M and L pigments which influence the spectral peak have also been extensively studied in vitro [8–11]. Asenjo and colleagues [11] showed that exactly seven changes are required to fully shift the spectrum from that of the shortest M to that of the longest L pigment and these changes are at amino acid positions 116, 180, 230, 233, 277, 285 and 309. The importance of the originally identified three positions [4] has been confirmed. The additional positions identified by in vitro studies contribute spectral shifts of less than 4 nm. They have been shown to influence the severity of color vision defects [12] and they presumably play a role in normal variations as well.

Taken in toto, these results from psychophysics, microspectrophotometry and molecular biology have made it clear that there are normal polymorphisms in the cone opsins, such as the one at position 180, which influence the spectral peak.

The remaining gap in knowledge concerns the character of the spectra of the pigment variants as they occur in the living human eye. The in vitro measures have been used effectively to determine which residues are involved in tuning the spectra. Those measures are particularly useful in evaluating relative differences in spectra; however, it is uncertain how accurately the

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Table 1
DNA Sequences

Donor	Amino acid position										
	65	111	116	153	171	174	178	180	230	233	236
P1	T	I/V	S/Y	L/M	V	AV	V	A/S	T/I	S/A	V/M
PI-A								A	I	A	M
PI-B								S/A	T	S	V
P2	T	I	S	L	V	A	I	S	I	A	M
P3	T	I	S	L/M	V	A	I	A	I	A	M
P4	T	I	S	L	V	A	I	A/S	I	A	M
P5	T	I	S	L	V	A	I	S	I	A	M

The amino acids present at each of the indicated positions in the L cone opsin were deduced from the DNA sequence. The single letter amino acid code is given (A, alanine; I, isoleucine; L, leucine; M, methionine; S, serine; T, threonine; V, valine; Y, tyrosine). The bold numbers indicate the amino acid substitutions that produce a spectral shift [11,10,4]. For some donors, two amino acids were encoded at individual positions and this is indicated by the single letter codes for the amino acids separated by a /.

measurements reflect the absolute spectrum of the cone pigment in situ. For example, two different in vitro methods give spectral peaks which disagree by >6 nm. The L pigment with serine at position 180 is reported to have peak sensitivities of 556.7 nm and 563 nm by Merbs and Nathans [10] and Asenjo et al. [11], respectively. It is difficult to know which more accurately reflects the spectral sensitivity of the intact cone photoreceptor. Further, the in vitro spectra are of limited utility because the spectrophotometric measurements are restricted to the regions of strong absorption near the peak sensitivity. In the present study, by recording the photocurrent of single cones, we have determined the spectral sensitivities of pigment variants in situ over a range of wavelengths, which extends into the spectral extremes. Wavelengths away from the middle of the spectrum (for example, the far reds) are used in diagnosing color vision defects and in examining variations in normal color matching. At these wavelengths individual differences are most pronounced and color defects can be most easily diagnosed. Spectral data, such as those presented here, will allow a quantitative analysis of the relationship between the spectral changes induced by naturally occurring amino acid substitutions and variations in normal and defective color vision as measured by color matching.

2. Methods

2.1. Tissue, cells and stimuli

Human retinas were obtained from the eyes of cornea donors or eyes removed due to craniofacial tumors. The mean retrieval time for the five cases in this study was 1.4 h. The ages and genders of the donors are listed in Table 2. Details of the tissue preparations have been given [13]. Briefly, the retina was isolated under dim red light in Lebovitz's L-15 medium and stored at 4°C in the dark. Experiments were performed on the same retina for three to four days, each experiment lasting about 3 h. For each experiment a 4×4 mm patch of retina was removed from cold storage and chopped under infra-red light to produce small pieces of retina about 50–100 nm on a side then warmed to near body temperature in a perfusion chamber. The circulating dark current of individual cones was recorded by drawing the outer segment into a suction electrode, 4 μm inner diameter. The photocurrent records and a stimulus trace were digitized at 2 ms intervals with hardware (MIO16) and software (LabView) from National Instruments (Austin, TX) and recorded on line. A stimulus set consisted of 5–30 responses to the same wavelength and intensity of light. Suction electrode recording has a

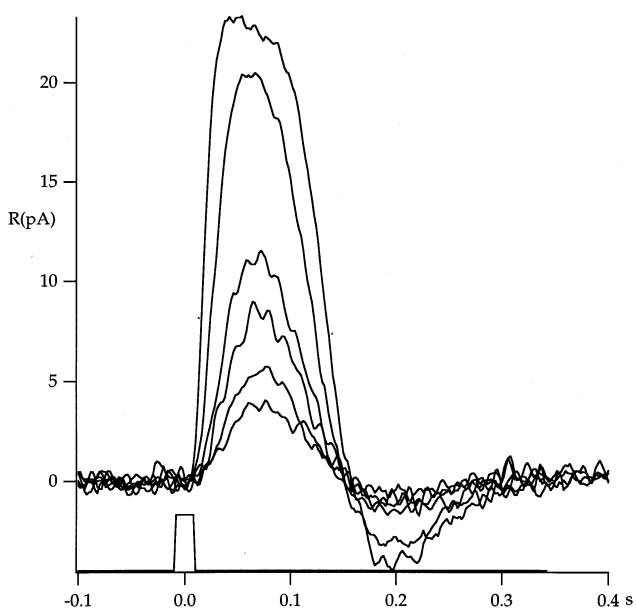

Fig. 1. Family of superimposed responses to brief flashes of 500 nm light. Current traces are averages of 5 to 10 responses; stimulus intensities varied from 642 to 33200 photons-μm^{-2}. A stimulus trace is given below the current traces. DC-150 Hz; T = 34°C.

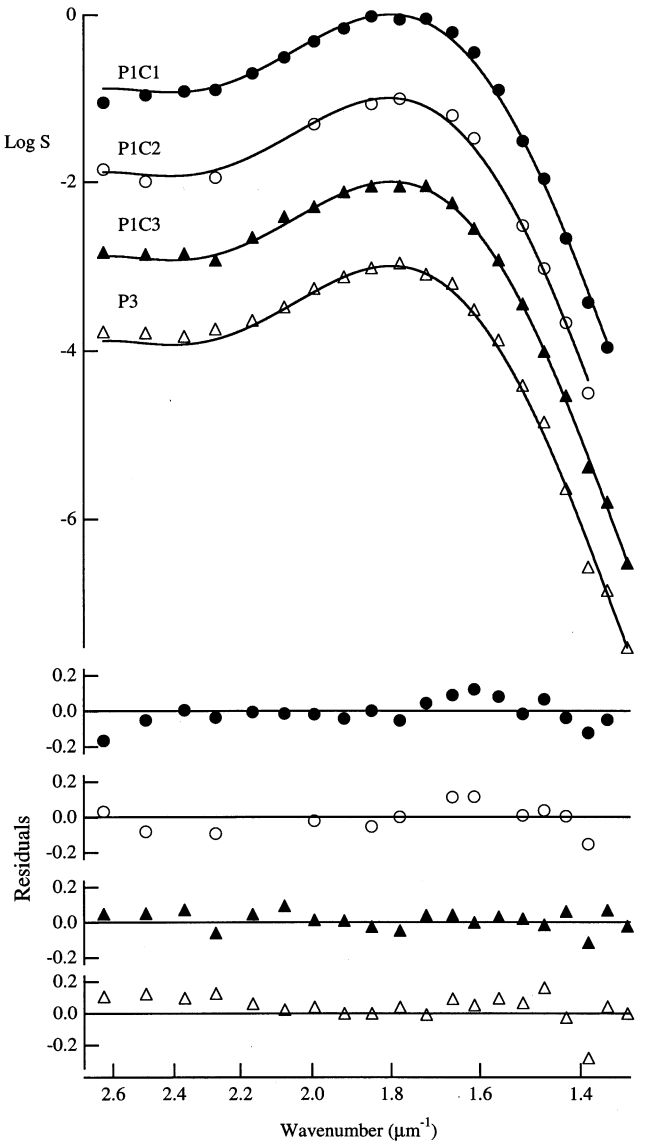

Fig. 2. Action spectra for four individual L cones from two donors. Different cells are represented by different symbols. Smooth curves represent individual fits of the Baylor polynomial to each data set. Results for each cell have been separated vertically for clarity. To show the error of the fits, residuals from the fits for each cell are plotted on an expanded sensitivity axis below, symbols for the residuals match the cell symbols identified in the upper graph. Maxima for these four cells fall between 554 and 556 nm, see Table 2.

resolution of about 0.25 pA when amplitude measurements are taken from the average of from 10 to 30 responses. The light bench focused a 440 μm diameter spot of light at the plane of the cells. The wavelength was controlled with 3-cavity interference filters (Andover, NH) with an average bandwidth of 10 nm. The light was attenuated by neutral density filters (Reynard, CA). Calibration of unattenuated light at each wavelength was performed daily with a photometer (Graseby Optronics, FL; Model 350).

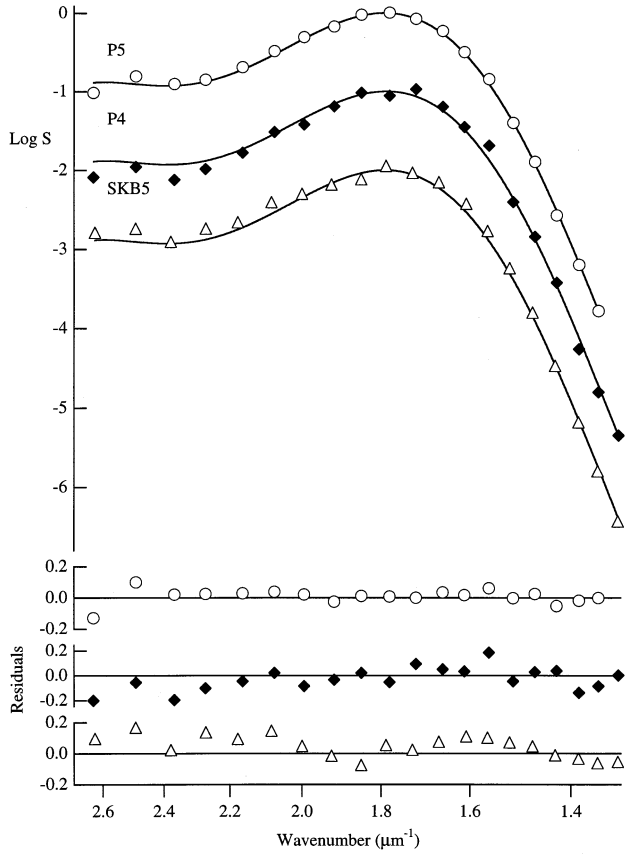

Fig. 3. Action spectra for two L cones from two donors (P4 and P5) and the mean spectral sensitivity function for five human L cones from a previous study (open triangles, SKB5) [16], error bars representing the standard deviation would fall within the symbol. Smooth curves are the resultant fits of the Baylor polynomial to each data set. Residuals for each fit are plotted below the main graph. Curves are separated vertically for clarity.

2.2. Measuring and fitting the action spectra of the cones

Spectral sensitivity of the visual pigments was estimated by measuring the action spectra using the criterion response method and the photocurrent responses

Table 2
Peak spectral sensitivities

Donor	Gender/age	Cell	λ_{max} (nm)	ΣX^2 (n)
P1	F/55	1	555.14	0.091 (19)
		2	554.10	0.071 (12)
		3	556.10	0.058 (20)
P2	M/75	1	558.08	0.345 (17)
		2	561.66	0.537 (20)
P3	F/71	1	555.66	0.192 (20)
P4	F/84	1	559.64	0.182 (21)
P5	F/79	1	558.22	0.040 (19)

The wavelength of maximum sensitivity was determined by fitting the Baylor polynomial to the complete action spectra for each cell (see Section 2). The final column gives the sum of the squared errors and, in parenthesis, the number of points in each curve.

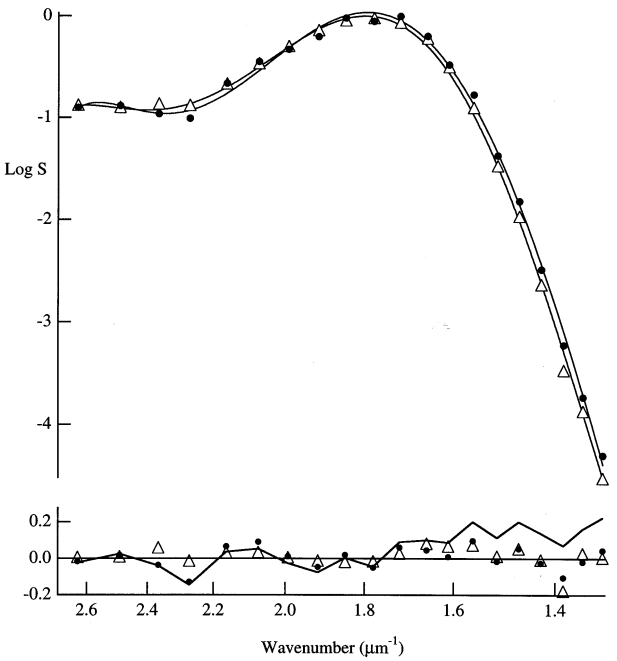

Fig. 4. Average spectral sensitivity curves for visual pigments grouped into two categories, the short L cone pigment and the long L cone pigment. Points are the average spectral sensitivity at each wavelength for four cells; the smooth curves are the Baylor polynomial optimized to each data set. The wavelengths of maximum sensitivity were 555.4 nm and 559.6 nm, respectively. Residuals from the fits are plotted with matching symbols on an expanded sensitivity axis in the lower graph. The straight line segments in the lower plot depict the residual when the best fit curve to the short L cone is subtracted from the average long L cone data.

of individual cones. The action spectrum was determined for up to 20 wavelengths at roughly 20 nm intervals between 380 nm and 760 nm. Spectral sensitivity was measured by adjusting the intensity of light at each wavelength to produce a criterion response of about 25% of the maximum current. The sensitivity at each wavelength was measured relative to a standard wavelength, (500 nm). Initially, for each cell, the complete intensity response function was determined at 500 nm. Subsequently, for each test wavelength, two or three light intensities were used to obtain current responses of 10 to 60% of maximum. Sensitivity measures at the standard wavelength were repeated after every two or three test wavelengths to avoid errors due to changes in the physiologic state of the cell or electrode seal.

Log relative sensitivity was plotted as a function of wavenumber. Prior to estimation of λ_{max} and to average the spectra across cells, the spectrum for each cell was shifted along the log sensitivity axis so that a sixth order polynomial fitted to the data points had a maximal value of log $S = 0$. The required vertical shift was fond to be insensitive to changes in either the order of the polynomial or the range of the wavelength fit. Thus this initial fitting of the spectral sensitivity curves was performed only to determine the maximum flash sensitivity.

To determine the wavelength of maximum sensitivity, or λ_{max} we presumed a common shape for all curves. The curve used was that described by Baylor and colleagues [14]; this curve has no theoretical basis, but was derived from a large data set (327 points of measured sensitivity from 16 L cones from 6 animals). Their sixth order polynomial was fit to a plot of Log sensitivity versus wavenumber (entered as μm^{-1}, plotted on a Log wavenumber axis). Each term of that polynomial has the form $a_n[\log(\lambda_{max}/\lambda \cdot 561)]^n$ where λ_{max} is the coeffficient that slides the polynomial curve along the wavelength axis to optimally match the data. The coefficients a_0 to a_6 are -5.2734, -87.403, 1228.4, -3346.3, -5070.3, 30881 and -31607, respectively [14]. Thus for each cell the spectrum was fit with only one free parameter, λ_{max} for that cell.

2.3. Molecular genetic analyses

Retinal samples for molecular genetic analyses usually consisted of a 4 mm by 20 mm strip of far peripheral retina, near the ora serrata. Larger and more central samples were also taken from the retinas of donors P1, P4 and P5. All samples were isolated with minimal RPE and vitreous and stored at -70°C. The analysis of the cDNA sequences was completed without knowledge of the results for the action spectra. Collaborators shared results when all data had been analyzed.

To isolate the nucleic acids, retinal tissue was placed in 200 μl of 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% SDS, 600 μg proteinase K and incubated for 8–12 hours at 55°C. The solution was gently extracted twice with phenol:chloroform in a 1:1 ratio and nucleic acids were ethanol precipitated from the aqueous phase. Retinal RNA was reverse transcribed and the resulting cDNA was used in the polymerase chain reaction (PCR) to selectively amplify the L pigment cDNA. Optimal amplification was obtained using Ampliwax gems in hotstart PCR with the XL PCR kit (Perkin–Elmer) and reaction volumes of 100 μl containing 1.4 mM magnesium acetate and 30 pmol of each primer. One primer (5′CCTTCGAAGGCCCGAATTA3′) annealed to the 5′ end of exon 2 and the other one (5′GCAGTACGCAAAGATCATCACC3′) annealed specifically to exon 5 of L pigment cDNAs [15]. Reactions were incubated at 94°C for 5 min, then subjected to 35 cycles of 94°C for 1 min, 59°C for 45 s and 72°C for 1 min. The reactions were then incubated at 72°C for 10 min.

The PCR products were reamplified with visual pigment cDNA specific primers that were tagged with the M 13–21 mer or reverse primer and both strands were directly sequenced with fluorescently labeled M13

primers in cycle sequencing using AmpliTaq FS (Perkin–Elmer) and an ABI 310 genetic analyzer. Reamplifications for sequencing were done with Amplitaq Gold (Perkin–Elmer) and two different primer pairs. One primer pair was used to amplify a DNA segment containing exons 2 and 3 and another primer pair was used to amplify exon 4. The first pair of primers was 5′TGTAAAACGACGGCCAGTCCTTCGAAGGCCCGAATTA, which anneals to the 5′ end of exon 2 and is tagged at the 5′ end with the M13-21mer sequence and 5′CAGGAAACAGCTATGACCCTGCTCCAACCAAAGATGG which anneals to the 3′ end of exon 3 and is tagged at the 5′ end with the M13 reverse primer sequence. PCRs with these primers were incubated at 94°C for 9 min, then cycled 35 times through the series 94°C, 59°C and 72°C for 45 s at each temperature and finally held at 72°C for 10 min, then used in sequencing. The second primer pair was 5′TGTAAAACGACGGCCAGTACTGGCCCCACGGCCTGAA, which is tagged with the M13-21mer and anneals to the 5′ end of exon 4 and 5′CAGGAAACAGCTATGACCTCGGATGGCCAGC CACACTT which is tagged with the M13 reverse sequence and anneals to the 3′ end of exon 4. PCRs with these primers were incubated at 94°C for 9 min, then cycled 35 times through the series 94°C, 61°C and 72°C for 45 s at each temperature and finally incubated at 72°C for 10 min and directly sequenced. Dye-primer chemistry was used here because the relative peak heights of two nucleotides at individual positions provides a semi-quantitative estimate of the relative amounts of the different species present in the sequencing reaction. Signal detection was possible above a noise level of about 20%, meaning that nucleotide ratios between about 3:1 to 1:3 could be assessed.

3. Results

Exons 2, 3 and 4 of the L pigment cDNAs encoded 11 amino acid positions that were dimorphic in the five donors of this study. Substitutions at four of these amino acids positions (116, 180, 230 and 233) produce spectral shifts [11,10,4] and thus specify spectral variants of the normal L pigment. The nucleotide sequences of the L pigment cDNAs expressed in each retina were used to deduce the amino acids specified at each of the 11 dimorphic positions, as shown in Table 1. For donors P1, P3 and P4 there are two amino acids encoded at specific positions, indicating that in the retinas from each of these donors, at least two different L pigment cDNAs were expressed. This is particularly important with regard to amino acid positions 116, 180, 230 and 233 since differences at these positions produce spectral subtypes of L pigment. The substitution at position 180 produces a spectral shift on the order of 7 nm and has been shown to account for the majority of the variability in normal color matching [5,15]. The substitution at position 116 produces a spectral shift of about 3 nm and the substitutions at 230 and 233 together produce a shift of about 3–4 nm [11,10]. In contrast, based on in vitro studies, it is thought that the dimorphism at amino acid position 153 is not spectrally active [11].

The molecular genetic results suggested that donors P2 and P5 each expressed only genes for L pigments with serine at position 180 (L_{ser180}). Donor P3 expressed only genes for L pigments with alanine at position 180 (L_{ala180}), Donors P1 and P4 each expressed both genes encoding L_{ala180} and L_{ser180} pigments. In addition to the dimorphism at position 180, the L pigment cDNAs from donor P1 encoded more than one amino acid at two other spectrally active positions, 230 and 233. The combinations of amino acids that occurred at positions 180, 230 and 233 in the L pigments in donor P1 were determined using PCR to selectively amplify subtypes of L pigment cDNAs based on sequence differences in exon 4, as described previously [15]. The sequence of exon 3 associated with each of the exon 4 sequences was determined. Donor P1 expressed genes for at least two different L_{ala180} pigments, one that specified isoleucine and alanine at positions 230 and 233, respectively and another that specified threonine230 and serine233 (P1-A and B, Table 1). Donor P1 also expressed a gene encoding a L_{ser180} pigment with threonine 230 and serine 233 (P1-B, Table 1).

Spectral sensitivity functions were derived from photocurrent recordings from individual human cones. Fig. 1 shows the superimposed responses recorded from a human cone to 20 ms flashes of 500 nm light of increasing intensity.

The spectral sensitivity results and best fitting template-curves for four cones from two donors are given in Fig. 2. Individual cells are represented by different symbols, the plots have been separated by one Log unit on the sensitivity axis for clarity. The smooth curves represent the Baylor Nunn and Schnapf polynomial (Baylor polynomial) which has been fit to individual cone spectra by moving the curve along the log wavenumber axis to minimize the sum of the squared error. The lower graph shows the residual error after the fitted curve has been subtracted from the data (symbols for residuals match the symbols for each cells' spectral data). The Log sensitivity axis of the residual plots has been magnified to demonstrate errors in the fit.

Fig. 3 shows similar plots of the two spectra obtained from L cones from patients P4 and P5. The third trace in Fig. 3 (Open triangles), labeled SKB5, is the mean spectral sensitivity of five human L cones published previously by Schnapf et al. [16]. The spectral sensitivity results for these five cells were re-analyzed individu-

ally using the same methods described for this paper. The individual spectral curves were noisier by a factor of about two for this older data (sum of squared errors); the individual peaks varied from 558.2 nm to 559.7 nm. The peak of the mean spectral sensitivity (Fig. 3 open triangles) was 558.6 nm.

A total of eight cells from five donors were examined, in which the cDNA sequences had also been determined. Each spectral sensitivity function was fit with the Baylor polynomial with λ_{max} as the only free parameter (see Section 2). The resulting maxima are listed in Table 2. The spectral peaks fell into two groups of four cells each. For one group, which we will refer to as 'long' L cones, the λ_{max} was greater than 558 nm. For the second group, which we will call 'short' L cones, the λ_{max} was equal to or less than 556 nm. A statistical test of the spectral peaks found significantly different population means for the long and short L cones ($P < 0.01$, two tailed t distribution).

The spectra measured for the L pigments from donor P3 fell into the 'short' class and the cDNA sequence indicated this donor expressed a typical L_{ala180} pigment [17]. For donors P2, P4 and P5, the spectral measurements indicated that the cones fell into the 'long' class of L pigments and all three donors expressed cDNA sequences encoding typical L_{ser180} pigments [17]. Donor P4 also expressed a cDNA encoding a typical L_{ala180} pigment, however, a cone containing this pigment would be expected to have a much shorter action spectrum than was observed.

The spectral measurements for donor P1 indicated that all three L cones examined fell into the short class. The cDNA sequence data indicated that donor P1 expressed multiple genes including two encoding L_{ala180} pigments (P1-A and B, Table 1). We assumed that all three cones for which action spectra were obtained expressed a typical L_{ala180} pigment (S116, A180, I230 and A233) for several reasons. First, the sequence data indicated that the predominant L pigment cDNA encodes S116. That is, the relative ratio of the nucleotide peaks at the position that determines whether S116 or Y116 is specified was 3:1, with the larger peak corresponding to the nucleotide that specified S116. Second, an L pigment with A180, T230 and S233 would be expected to have a spectrum that is 3–4 nm shorter than was observed [11,9]. Third, an L pigment with S180, T230 and S233 would be expected to have a spectrum that is 3–4 nm longer that was observed [11,9]. Thus, the spectra observed are most consistent with cone cells containing an L pigment with S116, A180, I230 and A233.

Averaging the sensitivities at each wavelength for the two groups yields prototypical spectral sensitivity curves for the two subtypes of human L cones based on the amino acid substitution of alanine for serine at position 180. In Fig. 4 the mean sensitivity for four 'short', L_{ala180} cones (open triangles) and four 'long', L_{ser180} cones (filled circles) are plotted. The smooth curves are the Baylor polynomial fits to the mean data; the two curves are separated by 4.2 nm. Residuals to the fits are plotted on the lower graph. If the best fitting curve for the L_{ala180} cones is subtracted from the average sensitivity values for the L_{ser180} cones a clearly poor fit is obtained; the residuals for this 'misfit' are shown by the line segments in Fig. 4 (lower plot).

4. Discussion

Previous studies [5,15] have shown that two major variants of L cone pigment in the human population differ by serine versus alanine at amino acid position 180. This amino acid difference appears to be responsible for the majority of variation in color matching among men with normal color vision. The results presented here represent the first direct, in situ measurement of the spectral separation produced by this amino acid dimorphism in L cone pigments.

Two donors studied here (P1 and P4) each expressed genes encoding both L_{ala180} and L_{ser180} pigments. For donor P4, the spectrum for the one L cone measured fell into the long class of L cone, consistent with the idea that the L cone for which spectra was measured expressed a gene for a L_{ser180} pigment. For donor P1, the spectral measurements for all L cones examined fell into the short class. Together the molecular genetic data and the spectral measurements are consistent with all three L cones expressing a typical L_{ala180} pigment (S116, A180, I230 and A233). The cDNA sequence data indicated that the predominant L pigment cDNA expressed specified S116. The spectral data are more consistent with a L_{ala180} pigment with S116, I230 and A233. Using the action spectra to determine the spectral sensitivity of a visual pigment makes use of the built in biological amplification of the phototransduction cascade. Essentially we measured the probability of photon capture presuming that all activity beyond that event is independent of wavelength. An advantage of this technique is that the range of measurements is limited only by the power of the optical system to deliver enough photons to overcome a low probability of photon capture. Thus action spectra can measure sensitivity over a range of from four to seven orders of magnitude. The process is laborious and far fewer wavelengths are sampled with this technique compared with reconstitution experiments [10,11], but the long wavelength end of the spectrum is more completely described using action spectra.

Precise determination of λ_{max} and the position of the spectral sensitivity function of all visual pigments is complicated by the relative flatness of the peak. Changes in log sensitivity of only 0.02 log units (5%)

from the peak represent shifts of 12 nm in wavelength. The use of the Baylor polynomial was intended to show the differences of the spectral sensitivity functions for the pigments based on different sequences by making use of the linear fall-off of sensitivity at long wavelengths. The differences between the pigment spectra are small but real and they agree with the predicted spectra based on sequence information.

When fitting the Baylor polynomial to the spectral sensitivity functions there was a systematic underestimate of sensitivity from 420 to 480 nm and from 620 and 680 nm. The discrepancies are clearly observed in the residual plots (Fig. 2, Fig. 4, lower plots). The same type of harmonic discrepancy is seen in the fit to the five long L cone spectra cones and their average (Fig. 3, SKB5 open triangles). The Baylor curve was not optimized for a fit near the top of the curve. It is useful, rather, in that it provides a fit of the entire extent of wavelengths measured for these cones (ca. 380–780) and it thus, allows good estimates of the relative differences in spectral peak among the cones. Its use also allows comparisons with the earlier primate (including human) electrophysiological spectral measurements. The Lamb equations [18] provide a good description of the long wavelength region of these measured spectra but they are not applicable to spectral points below 450 nm, which comprise a significant part of our data set. To get a better fit for wavelengths between 420–680 and thus a more accurate estimate of the spectral peak of theses cones an 8th order polynomial was fit to the 71 data points from four L_{ala180} cones[1]. This resulted in a curve with a maximum at 559.2 nm and the residuals from the fit showed no pattern of deviation and a 30% reduction in the sum of squared errors. A similar fit to the L_{ser180} cone data (76 data points) resulted in a curve with 563.4 nm as the wavelength of maximum sensitivity. These figures are in agreement with the absorption peaks of 563 nm for ser 180 and 556 nm for the ala l80 cones measured by Asenjo et al. [11].

The spectral peak of the L pigment can be influenced by amino acid changes encoded by exons 2, 3 and 4 of the pigment gene. In the normal population, serine and alanine occur with nearly equal frequency at position 180 [5,15] and that is the only variant amino acid position encoded by exon 3 that has been shown to produce a spectral shift [11]. Normal variations in spectrally-active, exon 4-encoded amino acids have been shown to occur but do so at a lower frequency than the position 180 dimorphism [5,15]. The frequency of normally occurring exon 2 encoded spectral variation is least certain, however, it appears that such variations occur with relatively low frequency [19]. These considerations would suggest that numerous spectral variants of the L pigment gene occur in the population considered to have normal color vision. However, the two spectral variants of the L pigment characterized here (559.2, 563.4) probably represent the two which occur most commonly in the human population.

[1] The form of the fitting equation is the same as was used by Baylor et al. [14] The coefficients for the optimized fit a_0 to a_8 are: 32.355, 1051.1, 10357, 38530,-11892, 565980, 1921200, 2827500 and -1615300 respectively.

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