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The thermal contribution to photoactivation in A2 visual pigments studied by temperature effects on spectral properties

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Abstract

Effects of temperature on the spectral properties of visual pigments were measured in the physiological range (5–28°C) in photoreceptor cells of bullfrog (*Rana catesbeiana*) and crucian carp (*Carassius carassius*). Absorbance spectra recorded by microspectrophotometry (MSP) in single cells and sensitivity spectra recorded by electroretinography (ERG) across the isolated retina were combined to yield accurate composite spectra from *ca.* 400 nm to 800 nm. The four photoreceptor types selected for study allowed three comparisons illuminating the properties of pigments using the dehydroretinal (A2) chromophore: (1) the two members of an A1/A2 pigment pair with the same opsin (porphyropsin *vs.* rhodopsin in bullfrog "red" rods); (2) two A2 pigments with similar spectra (porphyropsin rods of bullfrog and crucian carp); and (3) two A2 pigments with different spectra (rods *vs.* long-wavelength-sensitive (L-) cones of crucian carp). Qualitatively, the temperature effects on A2 pigments were similar to those described previously for the A1 pigment of toad "red" rods. Warming caused an increase in relative sensitivities at very long wavelengths but additionally a small shift of λ_{max} toward shorter wavelengths. The former effect was used for estimating the minimum energy required for photoactivation (E_a) of the pigment. Bullfrog rod opsin with A2 chromophore had $E_a = 44.2 \pm 0.9$ kcal/mol, significantly lower (one-tailed $P < 0.05$) than the value $E_a = 46.5 \pm 0.8$ kcal/mol for the same opsin coupled to A1. The A2 rod pigment of crucian carp had $E_a = 42.3 \pm 0.6$ kcal/mol, which is significantly higher (one-tailed $P < 0.01$) than that of the L-cones in the same retina ($E_a = 38.3 \pm 0.4$ kcal/mol), whereas the difference compared with the bullfrog A2 rod pigment is not statistically significant (two-tailed $P = 0.13$). No strict connection between λ_{max} and E_a appears to exist among A2 pigments any more than among A1 pigments. Still, the A1 → A2 chromophore substitution in bullfrog opsin causes three changes correlated as originally hypothesized by Barlow (1957): a red-shift of λ_{max}, a decrease in E_a, and an increase in thermal noise.

Keywords: Photoreceptor, Spectral sensitivity, Activation energy, Porphyropsin, Retinal

Introduction

Visual pigments form a large family of 7-TM receptors, where the protein (opsin) is covalently bound to a light-absorbing prosthetic group, the chromophore. Vertebrate pigments use either of two forms of chromophore: 11-*cis*-retinal, denoted A1, or 11-*cis*-3,4-dehydroretinal, denoted A2. A1 pigments are commonly called rhodopsins, and A2 pigments porphyropsins. In birds and mammals, only A1 has been found, whereas many fish and amphibian species can also utilize A2 or a mixture of both (Dartnall & Lythgoe, 1965; Bridges, 1972). Compared with A1, the A2 chromophore has an additional double bond in the β-ionone ring, a difference which *a priori* is expected to lower the energy barrier for isomerization (Bridges, 1956, 1967). The switch from A1 to A2 encountered in fishes and amphibians as seasonal or developmental changes (Dartnall et al., 1961; Bridges, 1964; Reuter, 1969; Allen & McFarland, 1973; McFarland & Allen, 1977) has two physiologically relevant effects. First, it red-shifts the wavelength of peak absorption (λ_{max}) of the visual pigment by typically more than 20 nm and broadens the absorbance spectrum (Dartnall & Lythgoe, 1965; Bridges, 1967; Bridges & Yoshikami, 1970; for review see Bridges, 1972; about spectral broadening, see Makino et al., 1999). Even partial A1 → A2 substitution strongly enhances sensitivity to long wavelengths. Second, the switch decreases the thermal stability of the pigment molecule. Porphyropsin in extract decays more rapidly than rhodopsin (Dartnall, 1955; Bridges, 1956, 1967; Williams & Milby, 1968), and this probably reflects the same underlying difference as the at least ten-fold higher rate of photon-like "dark" events (spontaneous pigment activations) observed in the receptor current of porphyropsin rods compared with rhodopsin rods of the bullfrog (Donner et al., 1990). Thus, the chromophore is an important determinant of the two most impor-

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tant functional properties of the visual pigment: spectral absorbance, which defines the visible range of electromagnetic radiation, and the rate of thermal activation, which sets an ultimate limit to visual sensitivity in darkness (Autrum, 1943; Barlow, 1956; Ashmore & Falk, 1977; Aho et al., 1988).

The absorbance spectra of visual pigments show subtle changes with temperature not only in extracts under extreme cooling (St. George, 1952; Yoshizawa & Horiuchi, 1969; Yoshizawa, 1972), but even *in vivo*. One effect is a small shift of λ_{max}, which at least in the A1 "red" rods of toad is clearly measurable even in the physiological temperature range (Ala-Laurila et al., 2002). Most interesting, however, is the relative increase in long-wavelength sensitivity with rising temperature (de Vries, 1948), which according to theory originally put forward by Stiles (1948) allows estimation of the minimum energy needed for photoactivation (E_a) of the pigment molecule (Denton & Pirenne, 1954; Srebro, 1966; Koskelainen et al., 2000). Ala-Laurila et al. (2002) showed that the temperature effect on the A1 pigment of toad "red" rods is in agreement with Stiles' theory as developed by Lewis (1955) and estimated E_a on this basis.

Here we use the same methods to study four other visual pigments *in situ* in photoreceptor cells, selected to allow three comparisons that elucidate the properties imparted by the A2 chromophore. The first comparison is between an A2 pigment and its A1 counterpart with the same opsin (bullfrog porphyropsin vs. rhodopsin rods). The second comparison is between two A2 pigments with similar absorbance spectra (porphyropsin rods of bullfrog vs. crucian carp). The third comparison is between two A2 pigments with different spectra (rods vs. L-cones of crucian carp). Thus, in the latter two comparisons the opsins are varying while the chromophore (A2) is the same.

The results are consistent with our earlier conclusion for A1 pigments that no unique and inevitable physical connection exists between λ_{max} and E_a (Koskelainen et al., 2000; Ala-Laurila et al., 2002). However, for the one opsin where all parameters are now available, the visual pigment of bullfrog "red" rods, the changes in λ_{max}, E_a, and thermal noise due to the chromophore substitution *per se* correlate qualitatively as predicted by Barlow (1957). The spectral red-shift achieved by switching from A1 to A2 is indeed associated with lowered activation energy and increased thermal noise.

Methods

Animals—preparations and recording

Crucian carp (*Carassius carassius*, body length *ca*. 7–15 cm) were caught in the autumn in a pond in SW Finland, kept in aquaria at 15°C on a 12-h light/12-h dark regime and fed with standard food for aquarium fish. Medium-sized bullfrogs (*Rana catesbeiana*, body length *ca*. 10 cm) were obtained from Carolina Biological Supply Co. (Burlington, NC). They were kept in either of two conditions, favoring or suppressing the development of significant proportions of A2 pigment in dorsal rods (Reuter et al., 1971). Porphyropsin-favoring conditions implied a 12-h light/12-h dark light regime at 15°C in basins with a white floor, lit from above by fluorescent tubes (Tsin & Beatty, 1980; Donner et al., 1990). The second, "rhodopsin" group was kept in natural daylight (light–dark cycle varying from 12-h/12-h to 18-h/6-h during the summer half-year in Helsinki) at 20°C in grey plastic basins with covers strongly attenuating the illumination. The pieces of retina used for A2 experiments were taken from a crescent-shaped field near the dorsal rim of the retina, where the A2 proportion is maximal containing up to 81–89% porphyropsin (Reuter et al., 1971; Donner et al., 1990). The pieces of retina for the A1 experiments were taken from the central retina near the optic disc, least likely to contain A2.

This work is based on two types of recordings. The spectral absorbance of visual pigments *in situ* was studied by microspectrophotometry (MSP) from the outer segments of isolated rods or cones. The spectral sensitivities of the corresponding photoreceptor types were determined electrophysiologically by ERG recording of mass photoresponses across the isolated, aspartate-superfused retina. MSP is superior for spectral characterization in the main absorbance band of the visual pigment, whereas electrophysiology is superior in the long-wavelength range where pigment absorbance is low (cf. Govardovskii et al., 2000).

All rod recordings were performed at two "physiological" temperatures (8.5 ± 0.5°C, "cold", C and 28.5 ± 0.5°C, "warm", W) from the same sample of isolated cells (MSP recordings) or the same retina (ERG experiments). The experiments on bullfrog porphyropsin rods further comprised MSP recordings at room temperature (21°C) used for determination of the A1/A2 ratio in each experiment by fitting of room-temperature spectral templates (Govardovskii et al., 2000). In crucian carp L-cones, ERG was recorded at three different temperatures: 5.0 ± 0.5°C, 15.0 ± 0.5°C, and 25.0 ± 0.5°C, whereas MSP was done only at room temperature (21°C).

Our conclusions are based on temperature-dependent changes in relative sensitivities in the long-wavelength range, that is, in the range studied by ERG. One requirement for this, however, is the availability of reliable measurements over an essentially temperature-invariant spectral domain to allow correct normalization of "warm" and "cold" data sets relative to each other. The MSP recordings provide such a reference, to which the long-wavelength ERG data could be anchored. The ERG spectra are much less reliable at shorter wavelengths due to several potential artifacts (Ala-Laurila et al., 2002).

For a detailed description of most aspects of the Methods, the reader is referred to Donner et al. (1988), Koskelainen et al. (1994, 2000), Govardovskii et al. (2000), and especially Ala-Laurila et al. (2002). Here we focus on a few special procedures required in the present work (1) for separating cone from rod responses in the crucian carp ERG; (2) for extracting pure A2 (porphyropsin) spectra from MSP recordings in bullfrog rods containing A1/A2 mixtures; and (3) for analysing the ERG data from crucian carp cones, where MSP data at the correct temperatures are not available.

Isolation of L-cone sensitivities from the ERG mass potential of the crucian carp retina

Even when synaptic transmission has been blocked by aspartate, the ERG signal recorded across the isolated retina is the sum of several (ohmic voltage) components, notably those due to photocurrents of more than one photoreceptor types and Müller cell (glial) currents elicited by changes in extracellular potassium near the photoreceptors. The glial contribution does not, however, distort spectral sensitivities as measured here (see Ala-Laurila et al., 2002).

Cone photoresponses were isolated from rod responses by a double-flash technique (Fig. 1), utilizing the fact that flash sensitivity is higher and response recovery slower in rods than in cones. A first, rod-saturating *conditioning* flash was followed after a fixed delay by a second, cone-stimulating *test* flash. The *test* flash was

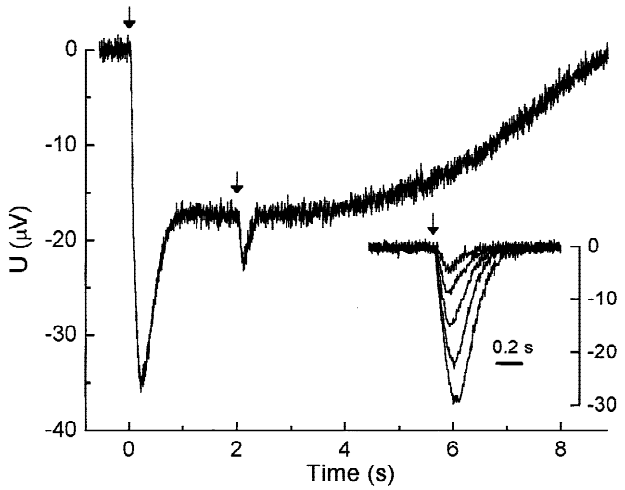

Fig. 1. The double-flash technique for isolating cone responses in the aspartate-isolated mass photoresponse recorded across the isolated retina of crucian carp. A rod-saturating blue (494 nm) *conditioning* flash at time $t = 0$ elicited a mixed response of rods and cones. When the cones had recovered but the rod response remained saturated, this was followed by a dim cone-stimulating *test* flash (at $t = 2$ s in the figure), the wavelength of which was varied. The trace is a single recording. Homogeneous 20-ms flashes, sampling frequency 200 Hz. The *Inset* shows a family of averaged (2–4) responses to flashes of increasing intensity at our reference wavelength for L-cone recordings, 702 nm (cone responses were low-pass filtered with 100-Hz cutoff). The responses were recorded in sequence just after the example trace shown in the main panel and with a similar protocol. Response families recorded at the reference wavelength were used for constructing the L-cone *I–R* functions needed for determining spectral sensitivities from small responses to test wavelengths (Koskelainen et al., 2000; Ala-Laurila et al., 2002). The temperature in the recordings shown was 15°C.

timed to occur when the cones had recovered, but while the rods still remained firmly saturated (this was assessed as described by Koskelainen et al., 1994). The required delay was 1–3 s (2 s in Fig. 1) depending on the temperature of the experiment. Michaelis-type saturation behavior was taken as indication that only one cone type was involved at the reference wavelength (702 nm). On these criteria, the response family to 702-nm flashes of increasing intensity shown in the inset of Fig. 1 is thought to represent the characteristics of essentially dark-adapted L-cones.

With test flashes of shorter wavelengths, two major artifacts may intrude. First, responses may contain contributions from other cone types, especially from the green-sensitive (M-) cones. According to our present MSP measurements, crucian carp retina contains four cone types, similar to those of the closely related goldfish, *Carassius auratus* (Marks, 1965; Stell & Hárosi, 1976; Palacios et al., 1998). Thus, we found (1) L-cones, $\lambda_{max} = 619$ nm, (2) M-cones, $\lambda_{max} = 531$ nm, (3) S-cones, $\lambda_{max} = 456$ nm, and (4) UV-cones, λ_{max} *ca*. 350–370 nm (all estimates obtained by fitting the A2 template of Govardovskii et al. (2000)). Second, as the retina was illuminated from the photoreceptor side, there could be substantial selective screening by the rod pigment, which has $\lambda_{max} = 526$ nm at room temperature. As we wished to deal with undistorted L-cone sensitivities, the study was limited to $\lambda \geq 680$ nm, where the sensitivity of the M-cone and the absorbance of the rod visual pigment both have dropped by more than 2 log units from peak.

Extraction of the porphyropsin spectrum from mixed A1/A2 spectra of bullfrog

Bullfrog "porphyropsin" rods in fact always have a mixture of A2 and A1 chromophores (Donner et al., 1990). The λ_{max} value of the A1 pigment has been measured with considerable accuracy (Reuter et al., 1971; Donner et al., 1990; Govardovskii et al., 2000). That of its A2 counterpart can be roughly defined by the general relation between rhodopsins and corresponding porphyropsins (Dartnall & Lythgoe, 1965; Hárosi, 1994), but greater precision was attained as follows. The good general templates available for A1 and A2 pigment spectra (Govardovskii et al., 2000) made it possible to determine what proportion of "mixed" absorbances was due to each by fitting the recorded spectrum with a linear combination of the two templates. Thus, we could purify the A2 absorbance component from spectra "contaminated" by A1 chromophore. The lower molar extinction coefficient of the A2 pigment need not be taken into account as long as the analysis is restricted to A1 and A2 *absorbance components* (which is how it will be used here unless otherwise stated). In terms of *numbers of visual-pigment molecules* underlying the absorbances, the proportions would of course be different, and this would be relevant, for example, if we wished to consider the amount of pigment-related thermal noise associated with a certain spectrum.

With respect to combining MSP and electrophysiology for studying rods with mixed chromophore content, two further points need to be emphasized. First, there is no significant difference in the quantum efficiency of bleaching between A1 and A2 pigments (see e.g. Dartnall, 1972). This means that the probability for an absorbed photon to isomerize the chromophore and trigger phototransduction is equal in the two. Second, response univariance holds at least for bullfrog rods, as studied here (Firsov et al., 1994). This means that the size or shape of the quantal response does not depend on whether it is initiated by a rhodopsin or porphyropsin molecule. The two variables by which we characterize spectral properties, outer-segment absorbance and flash sensitivity, are therefore directly proportional.

After each ERG experiment on bullfrog "porphyropsin" rods, pieces of the preparation from which the recordings had been made were transferred in darkness from the specimen holder to a Ringer dish and samples for MSP were prepared. MSP spectra were then recorded at room temperature (*ca*. 21°C), in each case from *ca*. 20 rods sampled from the retinal field just studied by ERG or from its immediate neighborhood. Thus, each ERG experiment is accompanied by an MSP spectrum recorded at the same temperature as those on which the Govardovskii et al. (2000) templates are based, the purpose here being accurate determination of the A1/A2 ratio. In part of the experiments, the ERG recordings were supplemented by two further MSP spectra recorded at the experimental temperatures of the ERG (*ca*. 8.5°C and at 28.5°C). Additional "porphyropsin-rod" MSP spectra at these temperatures were collected from samples of dorsal retina that had not been used in ERG experiments. In two control experiments, MSP spectra were recorded at all three temperatures from the same sample of 12 individual cells to assess to what extent variability of the A1/A2 ratio may cause spurious "temperature effects" in the main body of experiments, where different samples of rods were recorded at each temperature. The control experiments showed this possible source of error to be insignificant.

Besides knowledge of the A1/A2 ratio, the second prerequisite for extracting pure A2 spectra from mixed spectra at different temperatures is the availability of pure A1 spectra recorded at the

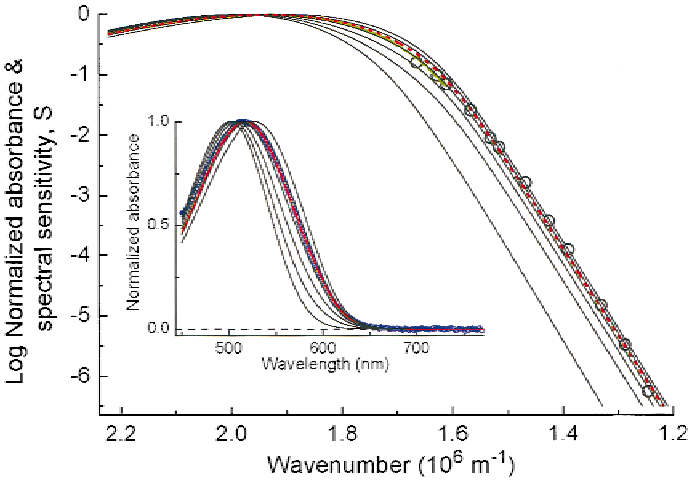

Fig. 2. The method for estimating the proportion of absorbance due to A2 visual pigment in bullfrog "porphyropsin" rods. *Main panel*: The black circles show logarithmic spectral sensitivities *vs.* wavenumber ($1/\lambda$) measured in a single ERG experiment at 28.5°C on a piece of dorsal bullfrog retina. The black curves show linear combinations of A1 and A2 templates (Govardovskii et al., 2000) corresponding to different A1/A2 absorbance proportions (0–100% in 20% steps). The dashed red line is the best-fitting template (corresponding to 71% A2 absorbance), carried over from the inset. The green trace is the synthetic MSP spectrum for 71% A2 at 28.5°C (see Text). *Inset*: The same templates (black curves) on linear scales, compared with an MSP spectrum averaged from 20 isolated cells taken from the same piece of retina and recorded at room temperature (21°C) (violet circles). The red line shows the best-fitting Govardovskii et al. (2000) template (least-square criterion over the interval 470–780 nm), corresponding to 71% A2 absorbance.

same temperatures. These were obtained from mid-ventral rods of "rhodopsin" retinas, containing no measurable amount of A2 chromophore as judged by template fitting. Assuming as small an admixture as 1–2% of A2 degraded the fits perceptibly compared with the pure A1 template. The A1 spectra were averaged across all experiments at each of the experimental temperatures.

Linear combinations of A1 and A2 templates, with the A2 percentage going from 0% to 100% in 20% steps, are shown in Fig. 2 (smooth continuous lines). The main panel presents the spectra on logarithmic ordinates against wavenumber, the Inset on linear scales. Especially the logarithmic format makes it obvious how strongly spectral sensitivities at long wavelengths are dominated by A2 even at quite low percentages of this chromophore (e.g. with only 20% A2, almost 90% of the sensitivity at wavelengths $\geq$ 650 nm is due to porphyropsin). The open black circles in the main panel give an example of a spectrum recorded in a single ERG experiment from the porphyropsin field of the retina at 28.5°C

The violet circles in the Inset shows the room-temperature MSP spectrum averaged from 20 rods from the same piece of retina that yielded the ERG data in the main panel. It lies between the templates for 60% and 80% A2, and the best fit corresponds to 71% A2 (red continuous line). The A1/A2 absorbance proportion in each ERG experiment was determined from room-temperature MSP in this way. For each experiment and temperature, we then recovered a pure A2 spectrum by subtracting the relevant proportion of the average A1 spectrum for that temperature from the mixed spectrum. The pure A2 spectra were averaged across experiments, separately for 8.5 and 28.5°C.

A high-quality "mixture" MSP spectrum was resynthesized for each experiment and temperature by summing the correct proportions of the *averaged* A2 and A1 spectra, and the relevant ERG data were anchored to this to yield a composite (MSP + ERG) spectrum. In the main panel of Fig. 2, the green trace is the synthetic MSP spectrum for 71% A2 at 28.5°C. The circles marking the ERG data have been joined to the MSP spectrum by least-square fitting based on the three ERG points (601, 615, and 621 nm) where the two data sets overlap. Before anchoring of the ERG data, the noisy long-wave tail of the MSP spectrum was smoothed by fitting of a second-order polynomial (cf. Ala-Laurila et al., 2002). Thus a composite "mixture" spectrum was obtained. For comparison, a linear combination of the relevant Govardovskii et al. (2000) A1 and A2 templates ($\lambda_{max,A1}$ = 501.6 nm) for 71% A2 is shown as a dashed red line in the main panel. Weighted subtraction of the A1 composite spectrum from the mixture composite spectrum gave a pure A2 composite spectrum at each temperature (see Fig. 3 below). Before subtraction, the A1 spectra were smoothed by fitting of a second-order polynomial to the long-wavelength tail (594–777 nm).

Analysis of the ERG data from crucian carp cones

Obtaining good enough MSP records from crucian carp cones to discern possible temperature effects near λ_{max} proved difficult. In

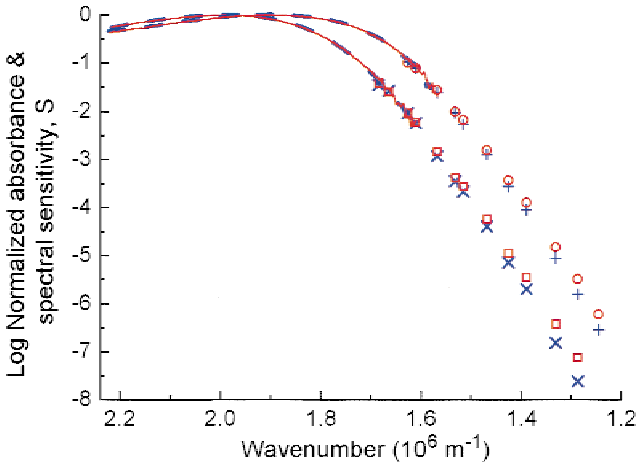

Fig. 3. Averaged composite spectra (log-normalized absorbance and log-normalized spectral sensitivity) of bullfrog rhodopsin and porphyropsin at 8.5°C ("cold") and at 28.5°C ("warm"). MSP data are shown by lines: blue dashed line (cold) and red continuous line (warm). ERG data are shown by blue and red symbols corresponding to "cold" and "warm" data, respectively: blue crosses and red squares (rhodopsin rods); blue plus signs and red circles (porphyropsin rods). The ERG data have been anchored with the least-square method to the corresponding MSP spectrum at the three shortest wavelengths, where two are seen to overlap (rhodopsin rods: 594–621 nm, porphyropsin rods: 615–638 nm). The porphyropsin spectra shown have been purified from mixed A1/A2 spectra (see Methods).

this case, therefore, we contented ourselves with MSP recordings at room temperature to get an accurate value for λ_{max}. The rest of the analysis was based on ERG data alone. ERG recordings were conducted at three temperatures (5, 15, and 25°C) over the long-wavelength domain where spectral distortion from M-cone responses and screening by rods can be neglected ($\geq$ 680 nm, see above). From the original spectra, *difference* spectra (15–5°C, 25–15°C, and 25–5°C) were formed without any previous normalizing assumptions (Ala-Laurila et al., 2002). Normalization is here deferred to *a posteriori* definition of a zero-difference baseline, which must be based on a wavelength domain where the relation between "warm" and "cold" sensitivities is supposedly temperature invariant. In fact, the experimental difference spectra did display a reasonably horizontal plateau (indicating constant warm/cold sensitivity ratio) abutting to the warming-induced rise at long wavelengths (see Fig. 5). The normalization of the difference spectra was therefore done by least-square fitting of the "plateau" data points to a zero line.

Results

Changing temperature affected the absorbance or sensitivity spectra of the A2 pigments (and, of course, the bullfrog A1 pigment as well) in two ways, consistent with results previously obtained in the A1 rods of the toads *Bufo marinus* and *Bufo bufo* (Ala-Laurila et al., 2002). First, there was a small shift in the wavelength of maximum absorption λ_{max}, averaging *ca.* −0.6 nm per 10°C temperature rise in the two A2 rod pigments studied here, bullfrog and crucian carp (data not shown). For these comparisons, the λ_{max} of each spectrum was determined by a polynomial fit to the data around peak (to absorbance values $A \geq 0.9$, see Ala-Laurila et al., 2002). The observed shift in λ_{max} is roughly consistent with interpolation to physiological temperatures of the shift observed upon cooling a carp porphyropsin extract from +15 to −190°C, where spectra published by Yoshizawa and Horiuchi (1969) and Yoshizawa (1972) indicate −0.4 nm per 10°C. A detailed consideration of the similar effect on λ_{max} of rhodopsins can be found in Ala-Laurila et al. (2002).

Second, there was an increase in relative sensitivities to long wavelengths, starting from some limit $\lambda_0 > \lambda_{max}$ and getting larger towards longer wavelengths. It is this effect that concerns us here, because it can be used for estimation of the minimum energy for photoactivation (E_a) of the pigments (Stiles, 1948; Lewis, 1955; Srebro, 1966; Koskelainen et al., 2000; Ala-Laurila et al., 2002). To measure the relative changes in long-wavelength sensitivities with sufficient accuracy, we used spectrophotometry and electrophysiology in the respective domains where each is superior and glued them together to form composite MSP + ERG spectra (see Methods, especially Fig. 2).

MSP spectra were collected from 98 (8.5°C) and 84 (28.5°C) rhodopsin rods (3 different animals) and from 97 (8.5°C) and 57 (28.5°C) porphyropsin rods from five different animals. ERG spectra were recorded from the ventral (rhodopsin) field in five retinas at 8.5°C and four retinas at 28.5°C and from the dorsal (porphyropsin) field in eight (8.5°C) and ten (28.5°C) retinas.

Fig. 3 shows composite "cold" (8.5°C) and "warm" (28.5°C) spectra for bullfrog rhodopsin and porphyropsin rods, plotted on logarithmic ordinates against wavenumber ($1/\lambda$). The lines trace the MSP data (dashed: cold, continuous: warm). For the A1 pigment, the MSP data are reproduced as such, whereas the A2 curves are conceptually purified spectra, where the contribution of some measured proportion of A1 present in the porphyropsin rods studied has been subtracted (see Methods). The symbols give the spectral sensitivities measured by ERG. Oblique crosses and squares give the averaged A1 data for the "cold" and "warm" conditions, respectively, upright crosses and circles give the averaged, purified A2 data for the two temperatures. The main point to observe is the divergence of "warm" and "cold" spectral sensitivities at long wavelengths.

Fig. 4A shows this interesting range on expanded scales. This format makes it easier to appreciate quantitatively the differences in the long-wavelength range and it also does justice to the fairly high precision of the measurements (note the SEM bars, which would have been obscured by the size of the symbols if drawn in Fig. 3). Panels B and C display the data for the two crucian carp pigments (B: 526-nm rods, C: 619-nm cones). Both these cell classes have A2 pigments with no measurable contribution from A1, and it is immediately evident that the temperature effects are smaller, emerging only at longer wavelengths compared especially with the bullfrog A1 pigment. On Stiles' (1948) theory, each warm–cold sensitivity difference can be used for an independent point estimate of the energy of photoactivation E_a (Denton & Pirenne, 1954; Srebro, 1966). Calculating E_a for each of the four pigments from the data shown in Fig. 4, with details as given by Koskelainen et al. (2000) and Ala-Laurila et al. (2002), we obtained the following values: bullfrog rhodopsin 46.5 ± 0.8 kcal/mol, bullfrog porphyropsin 44.2 ± 0.9 kcal/mol, crucian carp porphyropsin 42.3 ± 0.6 kcal/mol, and crucian carp L-cone pigment 38.3 ± 0.4 kcal/mol. The differences were statistically assessed by means of Student's *t*-test. When considering an A2–A1 pair with the same opsin, or two A2 pigments with significantly different λ_{max}, theory as well as earlier results (Barlow, 1957; Koskelainen et al., 2000) warranted that the zero hypothesis ("no difference") be confronted with one-tailed counter-hypotheses of the form: in case differences exist, the A2 pigment has lower E_a than its A1 pair, and the more red-sensitive pigment has lower E_a than the less red-sensitive one (with the same chromophore). The bullfrog A2–A1 difference is statistically significant ($P < 0.05$), as is that of crucian-carp L-cone versus rod pigment ($P < 0.01$). However, comparing the spectrally similar bullfrog and crucian-carp rod A2 pigments required two-tailed testing, and in this case the zero hypothesis could not be rejected ($P = 0.13$).

In Fig. 4, the data for all four pigments have been fitted with straight lines by weighted linear regression on $\log S - 1/\lambda$ scales (the weighting factor for each point being $1/\mathrm{SEM}^2$). The fits of the straight lines are rather acceptable overall and in qualitative agreement with Stiles' (1948) theory. Moreover, the regression coefficients (slopes) are on the order of magnitude predicted by the theory ($hc/kT \ln 10$),* although somewhat shallower. The values are as follows: bullfrog rhodopsin, 1.64×10^{-5} m (8.5°C) and 1.55×10^{-5} m (28.5°C); bullfrog porphyropsin, 1.66×10^{-5} m (8.5°C) and 1.57×10^{-5} m (28.5°C); crucian carp porphyropsin, 1.57×10^{-5} m (8.5°C) and 1.45×10^{-5} m (28.5°C); and crucian carp L-cone pigment, 1.99×10^{-5} m (5.0°C) and 1.77×10^{-5} m (25°C). These slopes lie between 70% and 89% of the Stiles prediction. A similar degree of agreement (76% of the theoretical value) was found by Ala-Laurila et al. (2002) in rhodopsin rods of *Bufo marinus*. Obviously, the theory further predicts that the *ratio*

*h = Planck constant, c = speed of light, k = Boltzmann constant, T = absolute temperature.

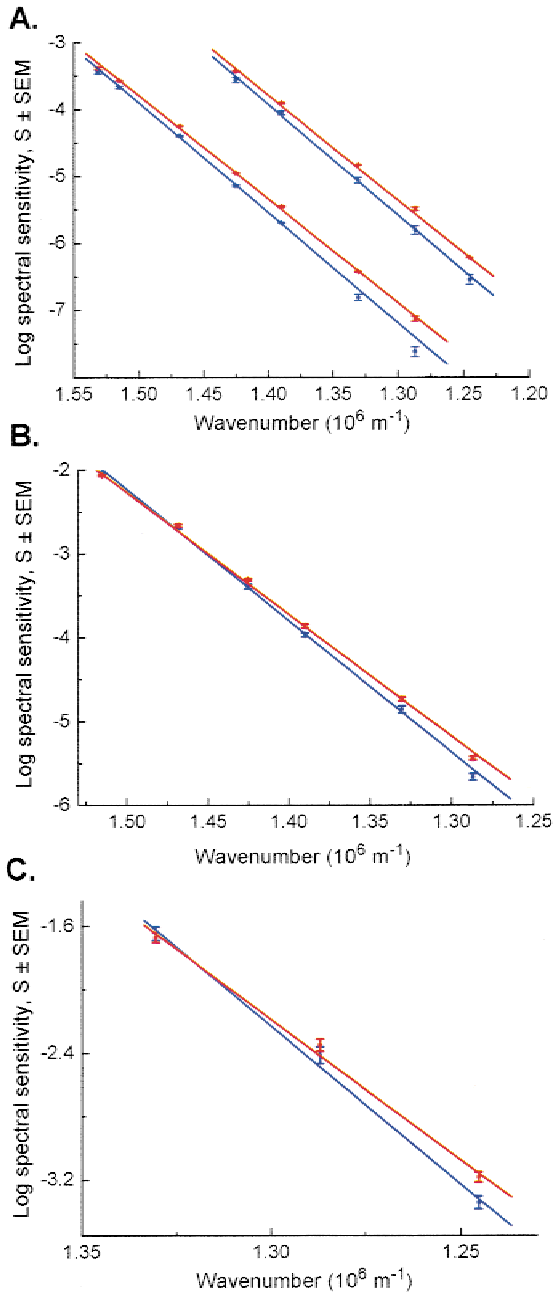

Fig. 4. The long-wavelength tails of ERG sensitivity spectra after anchoring to MSP. (A) Bullfrog rhodopsin and porphyropsin rods, (B) crucian carp rods, and (C) crucian carp L-cones. Spectral sensitivities at "cold" temperature (8.5°C in rod recordings, 5.0°C in crucian carp cone recordings) and at "warm" temperature (28.5°C in rod recordings, 25.0°C in crucian carp cone recordings) are shown by blue and red symbols, respectively. The error bars are SEMs. The data points are means of recordings from 4–10 retinas depending on the photoreceptor type and the temperature. The straight lines are fitted by weighted regression (weighting coefficient of each data point = $1/\mathrm{SEM}^2$). The wavelength intervals of the regression analysis, the regression slopes, and the coefficients of determination (r^2) are as follows: bullfrog rhodopsin rods (653 nm–777 nm), 1.64×10^{-5} m (8.5°C), $r^2 = 0.999$ (8.5°C), 1.55×10^{-5} m (28.5°C), $r^2 = 0.999$ (28.5°C); bullfrog porphyropsin rods (701 nm–803 nm), 1.66×10^{-5} m (8.5°C), $r^2 = 0.998$ (8.5°C), 1.57×10^{-5} m (28.5°C), $r^2 = 0.9995$ (28.5°C); crucian carp red rods (660 nm–777 nm), 1.57×10^{-5} m (8.5°C), $r^2 = 0.998$ (8.5°C), 1.45×10^{-5} m (28.5°C), $r^2 = 0.999$ (28.5°C); and crucian carp L-cones (750 nm–803 nm), 1.99×10^{-5} m (5.0°C), $r^2 = 0.997$ (5.0°C), 1.77×10^{-5} m, $r^2 = 0.996$ (25.0°C).

of slopes K_W and K_C at two temperatures T_W and T_C should be $K_C/K_W = T_W/T_C$. This factor is (301.7 K)/(281.7 K) = 1.071 for our experimental temperatures 8.5°C and 28.5°C and 1.072 for the temperatures 5.0°C and 25.0°C used in the L-cone experiments. The experimental ratios (K_C/K_W) obtained were 1.062 (bullfrog rhodopsin), 1.054 (bullfrog porphyropsin), 1.079 (crucian carp rhodopsin), and 1.123 (crucian carp L-cone pigment).

Fig. 5 summarizes the data in terms of the warm–cold *differences* in logarithmic relative absorbances or logarithmic relative sensitivities, plotted against $1/\lambda$. We shall term these functions Δ-spectra. They have been normalized with respect to a baseline that represents the absence of temperature effects (see Methods). Upward deviations from the zero line indicate relative increases in "warm" sensitivities.

According to Stiles' (1948) theory and its refinement by Lewis (1955), Δ-spectra should follow the baseline exactly up to the long-wavelength domain (beyond λ_0), where the relative increase in "warm" sensitivities sets in as a monotonical rise. In Lewis' formulation, the rise from baseline would start gently and gradually steepen (depending on the parameter m of the model) to reach finally the constant slope predicted by Stiles. In the range where the warm–cold differences have grown larger than 0.1 log units, the Lewis prediction converges with that of Stiles, envisaging straight lines of slope

$$K_\Delta = \frac{\mathrm{hc}(T_C - T_W)}{\mathrm{k}T_W T_C \ln 10}, \qquad (1)$$

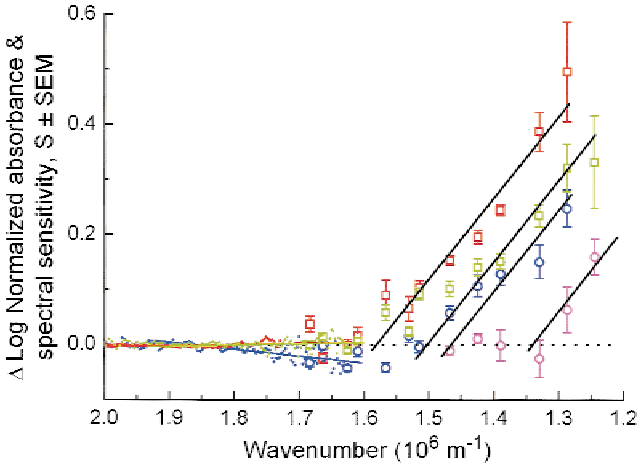

Fig. 5. Warm–cold difference spectra ($\Delta \log S = \log S_w - \log S_c$), showing the temperature effect on log relative spectral sensitivity and absorbance. MSP data are shown by small, filled symbols and ERG data are shown by larger open symbols (red: bullfrog rhodopsin rods, green: bullfrog porphyropsin rods, blue: crucian carp rods and pink: crucian carp L-cones). The dashed line shows the zero baseline determined by normalization of cold and warm MSP spectra to the same peak value. The error bars are SEMs. The straight black lines are constrained to have the slopes predicted by Stiles' model ($-1.47 \cdot 10^{-6}$ m and $-1.51 \cdot 10^{-6}$ m corresponding to the temperatures of rod and cone recordings, respectively) and have been positioned by a least-square criterion to fit the long-wavelength points for which $\Delta \log S \geq 0.1$ (for the crucian carp cones, both the data points that rise above baseline were accepted, implying loosening the criterion to $\Delta \log S \geq 0.06$). The points of intersection of the zero line and "Stiles" fits give the following graphical estimates of the activation energy: 45.2 kcal/mol (bullfrog rhodopsin rods), 43.0 kcal/mol (bullfrog porphyropsin rods), 41.9 kcal/mol (crucian carp rods), and 38.3 kcal/mol (crucian carp L-cones).

which for $T_C = 281.7$ K and $T_W = 301.7$ K gives $-1.47 \cdot 10^{-6}$ m. Straight lines with this slope have therefore been positioned for best fit to those data points that lie more than 0.1 log units above baseline. (A somewhat looser criterion was applied to crucian carp cones, see figure legend.) The lines are not inconsistent with the data, although the data as such would not, of course, suffice to determine the slopes with high precision. According to Stiles' original formulation, these lines intersect with the baseline at λ_0, thus defining the energy of photoactivation $E_a = hc/\lambda_0$. The E_a values derived in this way from Fig. 5 are 45.2 kcal/mol for bullfrog rhodopsin, 43.0 kcal/mol for bullfrog porphyropsin, 41.9 kcal/mol for crucian carp porphyropsin, and 38.3 kcal/mol for crucian carp L-cone pigment. These values are in fair agreement with the mean point estimates derived above (46.5, 44.2, 42.3, and 38.3 kcal/mol), but we would like to emphasize the primacy of the latter. While both methods are sensitive to limitations of the theory itself, the more simply "empiricist" point-by-point estimation is somewhat less vulnerable (see Ala-Laurila et al., 2002). One "non-Stiles" factor affecting both is the small temperature-induced shift in λ_{max}, interpreted by Ala-Laurila et al. (2002) to indicate slight variation of E_a itself with temperature (≤ 0.5 kcal/mol in the temperature range studied). Second, the slope of $\log S$ versus $\log 1/\lambda$ spectra continues to increase even in the temperature-sensitive wavelength domain where Stiles' theory would expect straight lines. Lewis (1955) accommodated this by introducing an extra parameter, the degrees of freedom of vibrational modes m. This, however, will enable a certain degree of tradeoff between parameters E_a and m when fitting Δ-spectra like those in Fig. 5. A direct comparison of the E_a values obtained for different pigments would require the assumption that m does not vary. Higher values of Lewis' m produce a gentler departure from baseline of the Δ-spectra, hence larger apparent E_a.

Discussion

The effects of changing chromophore from A1 to A2

The general objective of this and two previous studies (Koskelainen et al., 2000; Ala-Laurila et al., 2002) is to elucidate the relation between spectral absorbance and thermal stability of visual pigments. Barlow (1957) first hypothesized that the two be physically coupled, so that red-shifting the spectrum would always carry a cost in increased thermal noise. The basic idea is that both properties reflect the same underlying entity, the energy barrier for activation. A low barrier permits the pigment to be activated by low-energy ("red") photons, but also makes it susceptible to activation by thermal energy alone. As a universal law, the idea has been refuted theoretically as well as experimentally (Matthews, 1984; Goldsmith, 1989; Barlow et al., 1993, Koskelainen et al., 2000). Despite this, a significant *empirical correlation* exists between λ_{max} and thermal activation rate F in the sample of rod pigments where both properties have been measured (Firsov & Govardovskii, 1990; Fyhrquist, 1999). This is true also for λ_{max} versus dark noise in different cone types (Rieke & Baylor, 2000).

The chromophore switch from A1 to A2 in the same opsin, used by many species of fish and amphibians as a quick way of red-shifting spectral sensitivity, offers an attractively simple model for studying the correlation experimentally. Donner et al. (1990) measured dark noise in porphyropsin and rhodopsin rods of the bullfrog, finding that the *ca*. 25 nm red-shift of λ_{max} caused by the A1 → A2 switch is accompanied by a *ca*. 10-fold increase in the rate of thermal pigment activations F. In the present work the same pigment pair was characterized with respect to a third crucial parameter, the minimum energy for photoactivation E_a. We find that this parameter also differs between the two pigments in the direction envisaged by the Barlow hypothesis ($E_{a,A1} = 46.5$ kcal/mol is significantly larger than $E_{a,A2} = 44.2$ kcal/mol, $P < 0.05$). The ratio of the E_a values is close to the hypothesis' prediction based on the λ_{max} ratio ($E_{a,A2}/E_{a,A1} \approx 0.951$ *vs.* $\lambda_{max,A1}/\lambda_{max,A2} \approx 0.952$). However, the F ratio predicted from these activation energies is *ca*. 50 ($F_{A2}/F_{A1} = \exp((E_{a,A1} - E_{a,A2})/RT)$), that is, clearly larger than the ratio ~10 found by Donner et al. (1990). Thus, Barlow's (1957) simple hypothesis as such fails to account quantitatively for the difference in thermal stability even in pigments that differ only in the chromophore group. This is hardly surprising, as it has long appeared that the energy barriers for thermal and photic activation are different (Baylor et al., 1980; Barlow et al., 1993; Firsov et al., 2002). The molecular mechanisms underlying the correlation between λ_{max} and F must be more complex.

The opsin of bullfrog rods

Our conclusions about the effect of the chromophore in bullfrog rods are based on the assumption that the rhodopsin and the porphyropsin are both coupled to the same opsin (cDNA sequence published by Kayada et al., 1995). Several examples are known where the same photoreceptor cell can express more than one (even three) different opsins, either simultaneously or sequentially through different states of development (Shand et al., 1988; Wood & Partridge, 1993; Röhlich et al., 1994; Makino & Dodd, 1996). However, no case has been reported where the chromophore change would be linked to an opsin change. Fong et al. (1985) used isoelectric focusing to study varieties of rhodopsin in the leopard frog, *Rana pipiens* and the bullfrog, *Rana catesbeiana*. Although the frog rhodopsins, in contrast to bovine rhodopsin, separated into two forms, the authors did not find any differences between the porphyropsin and rhodopsin fields of the adult bullfrog retina, nor any differences associated with the A2 to A1 transition during metamorphosis. Moreover, the focusing pattern of bullfrog opsin was identical to that of the leopard frog, which has only A1 in the adult retina. Thus, we think that the possibility of an opsin difference between the porphyropsin and the rhodopsin of the bullfrog can be neglected.

The purity of pigments and spectra

Errors in the determination of E_a might arise if the spectra of other photoreceptors, not only those of bullfrog "porphyropsin" rods, in fact reflected some degree of A1/A2 mixture. We looked for possible contaminations by fitting in each case the "warm" absorbance spectra with A1 and A2 templates (Govardovskii et al., 2000) and we estimate that admixtures larger than a few percent of the other chromophore would have been detected. The bullfrog rhodopsin spectrum at 28.5°C was best fitted by a 100% A1 template with $\lambda_{max} = 501.7$ nm. This is in good agreement with the earlier result ($\lambda_{max} = 501.6$ nm) obtained by Govardovskii et al. (2000). The crucian carp spectra were best fitted by 100% A2 templates, with $\lambda_{max} = 526.3$ nm for the rod spectrum at 28.5°C and $\lambda_{max} = 619$ nm for the L-cone spectrum at 21°C. Likewise, the "purified" bullfrog A2 spectrum (at 28.5°C) was best fitted by a 100% A2 template with $\lambda_{max} = 525.2$ nm. This value is somewhat smaller than the value initially assumed for assessing the A1/A2 absorbance ratio in the original mixed spectrum, 527 nm based on

Firsov et al. (1994). We would like to emphasize, however, that the method is quite robust against small changes in assumed λ_{max}, especially when only spectra with high A2 proportion are accepted for analysis. The mixed spectra used here had A2 percentages of 60–70%. Then the "purifying" corrections are small in the crucial long-wavelength range and the E_a estimates for the A2 pigment would in fact change little even if no corrections were made.

A2 pigments compared with A1 pigments

The sample of E_a values for A2 pigments now available allows two conclusions. First, there is no unique and necessary connection between λ_{max} and E_a among these any more than among A1 pigments. Second, A2 pigments as a group have lower activation energies than A1 pigments. The ranges of E_a values encountered in the pigments hitherto studied by the same method are A2: 38.3–44.2 ($n = 5$) and A1: 44.3–50 kcal/mol ($n = 7$) (Srebro, 1966; Koskelainen et al., 2000; Ala-Laurila et al., 2002). Although the highest A2 value (bullfrog porphyropsin) and the lowest A1 value (*Bufo marinus* rhodopsin) virtually coincide at 44.2–44.3 kcal/mol, the ranges remain nonoverlapping.

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