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On the relation between the photoactivation energy and the absorbance spectrum of visual pigments

Petri Ala-Laurila [a,*], Johan Pahlberg [b], Ari Koskelainen [a], Kristian Donner [b]

[a] Laboratory of Biomedical Engineering, Helsinki University of Technology, P.O. Box 2200, Rakentajanaukio 2C, FIN-02015 HUT, Finland
[b] Department of Biosciences, University of Helsinki, P.O. Box 65, Viikinkaari 1, FIN-00014, Finland

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Abstract

We relate the collected experimental data on the minimum energy for photoactivation (E_a) to the wavelengths of peak absorbance (λ_{max}) of 12 visual pigments. The E_a values have been determined from the temperature-dependence of spectral sensitivity in the long-wavelength range. As shown previously, the simple physical idea $E_a = \text{const.} \times (1/\lambda_{max})$ (here termed the Stiles–Lewis–Barlow or SLB relation) does not hold strictly. Yet there is a significant correlation between E_a and $1/\lambda_{max}$ ($r^2 = 0.73$) and the regression slope obtained by an unbiased fit is 84% of the predicted value of the best SLB fit. The correlation can be decomposed into effects of A1→A2 chromophore change and effects of opsin differences. For a chromophore change in the same opsin, studied in two A1/A2 pigment pairs, the SLB relation holds nearly perfectly. In seven pigments having different opsins but the same (A2) chromophore, the correlation of E_a and $1/\lambda_{max}$ remained highly significant ($r^2 = 0.87$), but the regression coefficient is only 72% of the best SLB fit. We conclude that (1) when the chromophore is exchanged in the same opsin, the λ_{max} shift directly reflects the difference in photoactivation energies, (2) when the opsin is modified by amino acid substitutions, λ_{max} and E_a can be tuned partly independently, although there is a dominant tendency for inverse proportionality. In four (A1) rhodopsins with virtually the same λ_{max}, E_a varied over a 4.5 kcal/mol range, which may be taken as a measure of the freedom for independent tuning. Assuming that low E_a correlates with high thermal noise, we suggest that the leeway in $\lambda_{max} - E_a$ coupling is used by natural selection to keep E_a as high as possible in long-wavelength-sensitive pigments, and that this is why the opsin-dependent $E_a(1/\lambda_{max})$-relation is shallower than predicted.

Keywords: Photoreception; Rhodopsin; Spectral sensitivity; Activation energy; Thermal noise

1. Introduction

Visual pigments differ in two functionally important properties, spectral absorbance and thermal stability. Both are determined by the interaction of the covalently bound prosthetic group, the chromophore (in vertebrates, 11-*cis* retinal, A1, or 11-*cis*-3,4-dehydroretinal, A2) with the apoprotein, the 7-transmembrane receptor opsin. The first property, described by the absorbance spectrum, defines the wavelength band of electromagnetic radiation available for vision. Although modelling of shifts in spectral absorbance has made great advances in recent years (see e.g. Kochendoerfer, Lin, Sakmar, & Mathies, 1999; Takahashi & Ebrey, 2003), no comprehensive physical model exists that would enable prediction of spectral or thermal properties from molecular structure. The second property, thermal stability, here refers to the inverse of the probability that the pigment be activated by thermal energy alone, triggering a visual signal in the absence of light. This is a rare event, especially in rod pigments, where a molecule has a mean lifetime of ≈3000 years. Yet, among the 10^9 or so molecules in a rod cell, it will typically occur about once per minute (Baylor, Matthews, & Yau, 1980; Donner, Firsov, & Govardovskii, 1990; Firsov, Donner, & Govardovskii, 2002; Firsov & Govardovskii, 1990). In a cone cell, the rate is higher by ca. four orders of magnitude on average (Barlow, 1957; Donner, 1992; Rieke & Baylor, 2000; Schnapf, 1990). Such randomly occurring "false" activations will constitute a noise that is

* Corresponding author. Address: Department of Physiology and Biophysics, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118-2526, USA. Tel.: +1-617-638-5336; fax: +1-617-638-4273.
E-mail address: pal@iki.fi (P. Ala-Laurila).

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indistinguishable from that due to photon fluctuations and may set the absolute limit to the light sensitivity of the visual system (Aho, Donner, Hydén, Larsen, & Reuter, 1988; Autrum, 1940; Barlow, 1956).

To allow the highest possible visual sensitivity in a given light environment, spectral absorbance should be tuned for maximal quantum catch and the occurrence of spontaneous activations should be minimized. If spectral and thermal properties were independent, evolution would in principle have a straightforward task. If, however, there is some degree of coupling between the two, maximizing visual sensitivity becomes a question of striking an optimal balance between high quantum catch and low noise (maximizing the signal-to-noise ratio). Inspired by Stiles' (1948) theory for explaining the shape of the long-wavelength decline of visual spectra, Barlow (1957) suggested that pigments tuned to absorb long-wavelength light, i.e. low-energy photons, must have a low energy barrier for activation and therefore a high probability for activation by thermal energy alone (cf. de Vries, 1949). This would explain e.g. the seemingly paradoxical fact that rod pigments, designed for dim-light vision, are very often blue-shifted from the spectral position that would give the best quantum catch.

Barlow's (1957) hypothesis effectively implies that both spectral and thermal properties reflect a common underlying "activation energy" (E_a), i.e., the minimum energy required to take the molecule over the barrier from the inactive to the active conformation. In the original formulation, this energy was assumed to be equal to the photon energy at the wavelength of peak absorbance, λ_{max}, thus $E_a = hc/\lambda_{max}$. Lewis (1955) argued, however, that the just-sufficient photon energy may rather be represented by some longer wavelength, $\lambda_0 > \lambda_{max}$. We combine these insights into what we term the "Stiles–Lewis–Barlow (SLB) hypothesis", stating that E_a is a constant fraction of the photon energy at the wavelength of peak absorbance, i.e., $E_a = A \times hc/\lambda_{max}$, where A is a constant <1. Denoting $A = \lambda_{max}/\lambda_0$, the SLB relation can be written in the useful form $E_a = hc/\lambda_0$ (see Fig. 1).

The purpose of this paper is to study the dependence between E_a and λ_{max} using all available data. We have now estimated the photoactivation energy E_a of 12

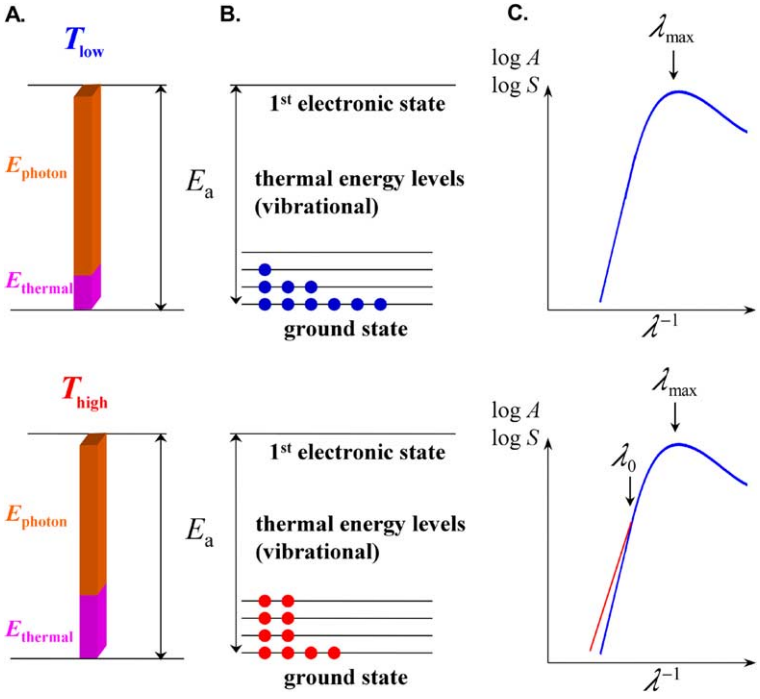

Fig. 1. How temperature affects the probability that photons of different wavelengths trigger visual excitation according to Stiles' (1948) theory. The top row of panels represents a lower temperature ("cold") and the bottom row a higher temperature ("warm"). (A) Photons with energy less than E_a can activate the pigment molecule only if supported by a sufficient amount of molecular thermal energy. The higher the thermal energy level of the pigment molecule, the smaller is the photon energy required for the activation threshold to be reached, thus the longer the wavelength of light that can still trigger a visual signal. (B) The relative proportion of rhodopsin molecules on higher thermal energy levels increases as temperature is raised. Stiles assumed that this can be described by the Boltzmann distribution. Lewis (1955) argued that a distribution derived by Hinshelwood (1940) is more appropriate for a complex molecule like rhodopsin. Under both models, the decline of log visual sensitivity as function of $1/\lambda$ in the long-wavelength ("thermal") domain will approach a straight line as a limiting value. (C) Absorbance, A or sensitivity, S spectra on logarithmic ordinate and $1/\lambda$ abscissa. The slope of the final straight line that describes the long-wavelength limb decreases as temperature is raised because of the increase in the proportion of rhodopsin molecules with enough thermal energy to enable activation by these low-energy photons. The effect starts at a limiting wavelength λ_0, where the photon energy is equal to E_a. The activation energy can be obtained by measurement of the magnitude of the temperature effect at wavelengths $\lambda > \lambda_0$. Under the SBL hypothesis (see text), the ratio λ_{max}/λ_0 is constant for all pigments.

visual pigments from measurements of how long-wavelength sensitivity changes with temperature (Ala-Laurila, Albert, Saarinen, Koskelainen, & Donner, 2003; Ala-Laurila, Saarinen, Albert, Koskelainen, & Donner, 2002; Koskelainen, Ala-Laurila, Fyhrquist, & Donner, 2000; cf. Srebro, 1966). Initially, our main conclusion was that the SLB hypothesis fails in a strict sense: the same E_a may be associated with quite different λ_{max} and vice versa (Koskelainen et al., 2000). However, the larger data set now assembled allows us to look for underlying trends beneath the (very substantial) inter-pigment variation. What emerges is an intriguing approximation to the SLB relation: E_a and $1/\lambda_{max}$ do correlate significantly, but on the whole the regression is not quite as steep as predicted. Only in the particular case where the λ_{max} shift is produced by a chromophore change does the SLB relation hold quantitatively to within the experimental error limits. We suggest that SLB coupling of E_a and $1/\lambda_{max}$ may be viewed as an underlying physical "default assumption" and that departures reflect a limited freedom for selective, independent tuning of the two. The quantitative relation between E_a and λ_{max} derived here helps us to understand the interdependence of λ_{max} and thermal noise in photoreceptor cells (cf. Section 4 and Ala-Laurila, Donner, & Koskelainen, 2004).

2. Materials and methods

The experimental data stem from microspectrophotometric and electrophysiological measurements on 10 vertebrate and two invertebrate visual pigments in situ in photoreceptor cells, where each cell type was studied at two or several different temperatures. The vertebrate pigments represent rods of *Bufo bufo*, *Bufo marinus*, *Rana temporaria*, *Rana catesbeiana*, *Xenopus laevis* and *Carassius carassius*, and L-cones of *Rana temporaria* and *Carassius carassius*. The chromophore of the *Xenopus laevis* and *Carassius carassius* pigments was A2, whereas the rod pigment of *Rana catesbeiana* and the L-cone pigment of *Rana temporaria* were studied both in their A1 and A2 versions and all the other vertebrate pigments had A1. The two invertebrate pigments are the A2 visual pigments of two Finnish populations of the Crustacean *Mysis relicta* having different spectral sensitivities, one from the Baltic Sea and the other from a lake with strongly humus-stained water (Lindström, 2000; Pahlberg et al., in preparation).

Fig. 1 provides a heuristic illustration of those aspects of Stiles' (1948) theory that concern us here. Why should temperature have an effect on long-wavelength sensitivity, and how can E_a be deduced from this effect? For detailed descriptions of the methods and the theoretical background, the reader is referred to Ala-Laurila et al. (2002, 2003).

The top panels show the situation at lower temperature, the bottom panels the situation at higher temperature. The leftmost pair of panels (A) illustrates the energy threshold for activation and how, at long wavelengths, the insufficient photon energy (red) must be supplemented by thermal energy (violet) of the visual-pigment molecule for the threshold to be reached. At higher temperature, activations based on relatively greater proportions of thermal energy (hence lower-energy photons) will be more frequent. The middle pair of panels (B) shows why this is so. The fraction of rhodopsin molecules that occupy higher thermal energy levels increases with warming. At higher temperatures a low-energy photon is more likely to encounter a molecule that has at least the required supplement of thermal energy. The rightmost pair of panels (C) shows how this appears in absorbance or sensitivity spectra. The increased probability for a low-energy photon to find a rhodopsin molecule with sufficient internal energy will be evident as a rise in relative sensitivity to such (long-wavelength) photons, as shown by the red limb of the "warm" spectrum (cf. de Vries, 1948). The wavelength domain where temperature effects will appear is limited from below by a wavelength λ_0, where the photon energy is equal to E_a. Photons with $\lambda < \lambda_0$ have enough energy in themselves and can draw no advantage from a raised temperature. Neglecting other factors, spectra should thus be temperature-invariant at $\lambda \leqslant \lambda_0$ but show raised "warm" sensitivities for $\lambda > \lambda_0$. The SLB hypothesis implies that the ratio λ_0/λ_{max} be constant across all visual pigments.

Obviously, E_a can in principle be obtained just by estimating λ_0. Due to several technical as well as theoretical complications (discussed in detail by Ala-Laurila et al. (2002, 2003)), obtaining a good estimate requires measurement of the temperature effect at several wavelengths $\lambda > \lambda_0$. The procedures for analysing spectra and determining λ_{max} and E_a from them are described in the respective experimental articles (Ala-Laurila et al., 2002, 2003; Govardovskii, Fyhrquist, Reuter, Kuzmin, & Donner, 2000; Koskelainen et al., 2000; Pahlberg et al., in preparation).

Statistics. The data, expressed as E_a vs. $1/\lambda_{max}$, were analyzed by weighted linear regression and correlation, the relative reliability of the respective points being taken into account by giving each the weighting factor $1/\mathrm{SEM}^2$ of the E_a estimate (SEM = standard error of mean). The one free parameter of the the SLB relation (its slope), was fixed by weighted linear regression (weight $1/\mathrm{SEM}^2$), whereby the regression line was constrained to run through the origin (0, 0). The ratio of the observed slope to that of the original Barlow (1957) relation, $d(E_a)/d(1/\lambda_{max}) = hc \approx 28,600$ nm kcal/mol, yields λ_{max}/λ_0, which according to the SLB hypothesis has to be constant for all pigments.

3. Results

Fig. 2 plots the activation energies E_a against wavenumber ($1/\lambda_{max}$) for all the pigments studied. The solid red line has been fitted to the data points by weighted linear regression (see Section 2). The equation of the line is

$$E_a = 7.10\ \text{kcal/mol} + 19,800\ \text{nm kcal/mol} \cdot \frac{1}{\lambda_{max}}\ (\text{nm}^{-1}). \quad (1)$$

The coefficient of determination $r^2 = 0.73$. In other words, 73% of all variation in E_a is "explained" by the correlation with $1/\lambda_{max}$. The dashed blue line shows the SLB relation, i.e., the best-fitting line constrained to pass through the origin. Obviously, the deviation between the two lines is rather modest (the best-fitting slope is 84% of the SLB slope), and in fact the SLB relation as such would explain 70.6% of the variation in E_a. The SLB slope (23,700 nm kcal/mol) indicates $\lambda_{max}/\lambda_0 = 0.829$, so that e.g. a rhodopsin at 500 nm typically would have $\lambda_0 = 603$ nm and $E_a = hc/\lambda_0 = 47.4$ kcal/mol. A further way of quantitating how much the experimental data depart from the SLB relation is to ask what confidence intervals would accommodate the latter. The narrowest confidence limits, shown by the green dotted lines in the figure, correspond to the probability level $P = 0.62$. Thus, even if the SLB relation were in fact the exact underlying "law", more than every third random sample (38%) of 12 pigments would yield a regression line that deviates by at least as much as observed just by chance.

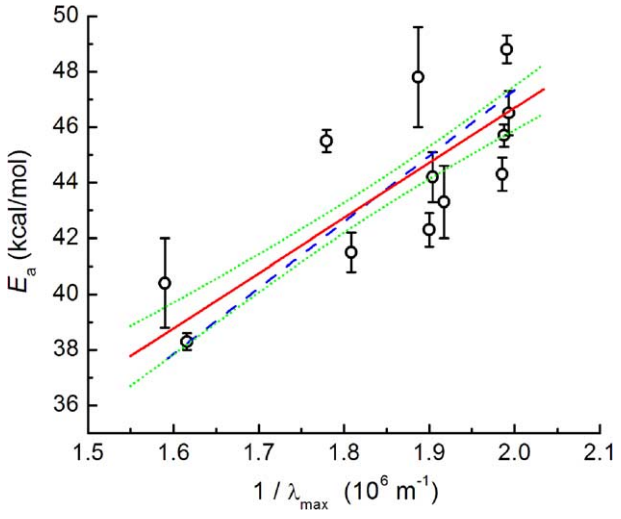

Fig. 2. The activation energy E_a plotted as function of $1/\lambda_{max}$ for the 12 visual pigments studied. The red line has been fitted by weighted linear regression to all data points (Eq. (1) in the text). The SEMs of E_a are shown as error bars (the errors in the determination of λ_{max} are negligible). The origins of the pigments are (from left to right, in order of increasing $1/\lambda_{max}$): (1) L-cones of *Rana temporaria* tadpoles (A2), (2) L-cones *Carassius carassius* (A2), (3) L-cones of adult *Rana temporaria* (A1), (4) *Mysis relicta* (lake population) (A2), (5) *Mysis relicta* (sea population) (A2), (6) rods of *Carassius carassius* (A2), (7) porphyropsin rods of *Rana catesbeiana* (A2), (8) rods of *Xenopus laevis* (A2), (9) rods of *Bufo marinus* (A1), (10) rods of *Rana temporaria* (A1), (11) rods of *Bufo bufo* (A1), (12) rhodopsin rods of *Rana catesbeiana* (A1). The chromophore is given in brackets. The dashed blue line shows the best-fitting SLB relation (passing through the origin and the weighted mean of the data points). The weighting factor in all cases is $1/\text{SEM}^2$ of the E_a estimate. The dotted green lines delimit the 62% confidence interval of the regression line. They represent the narrowest confidence limits that encompass the SLB line. For further details, see text.

The variation in E_a and λ_{max} arises from two different types of molecular modifications: chromophore change and opsin change. The switch between A1 and A2 chromophore is a very simple molecular event compared with the entire range of possible modifications available if any number of the 360 or so amino acid residues in the protein may be changed. E.g., under a chromophore switch the interaction of the protonated Schiff-base and the counterion remains the same. Thus it might be expected to have much more standardized effects. In Fig. 3, the effects of chromophore and opsin differences have been separated.

Panel A isolates the two pigment pairs (four data points) where measurements have been made with both the A1 and the A2 chromophore in the same opsin (*Rana catesbeiana* rods, RCA1 and RCA2, and *Rana temporaria* L-cones, RTA1 and RTA2). Shown are the best-fitting SLB lines, corresponding to $\lambda_{max}/\lambda_0 = 0.814$ (*Rana catesbeiana*, green line) and $\lambda_{max}/\lambda_0 = 0.894$ (*Rana temporaria*, blue line). Obviously, the deviations from the slopes that would obtained by simply connecting the two points of each pair (not shown) are insignificant, amounting to ≈0.5%. Thus the $E_a - 1/\lambda_{max}$-relation obtained from pigments differing only in the nature of the chromophore is in close agreement with the SLB hypothesis.

In panel B, all pigments with A2 chromophore but different opsins have been selected. Here, the regression line (shown in red), having slope 16,700 nm kcal/mol ($r^2 = 0.87$), differs more strongly from the SLB relation (blue dashed line, slope 23,300 nm kcal/mol). The tightest confidence limits accommodating the SLB line (green dotted lines) correspond to the probability level $P = 0.12$. Thus it is not possible even in this case to reject the SLB hypothesis as the true underlying trend on purely statistical grounds. Yet we think it is significant that a red-shift of λ_{max} appears, on average, to be accompanied by a *less strong* decrease in E_a than predicted (see Section 4).

For A1 pigments we do not have sufficient data to identify larger trends. The spectral peaks of four out of our five A1 pigments lie within the narrow interval 501–503 nm. Instead, these four E_a values for pigments with nearly the same λ_{max} allow us to make a cautious estimate of the extent to which E_a and λ_{max} can be tuned independently. The SD is 1.9 kcal/mol, or 4% of the average E_a, and the variation range is 4.5 kcal/mol, i.e.,

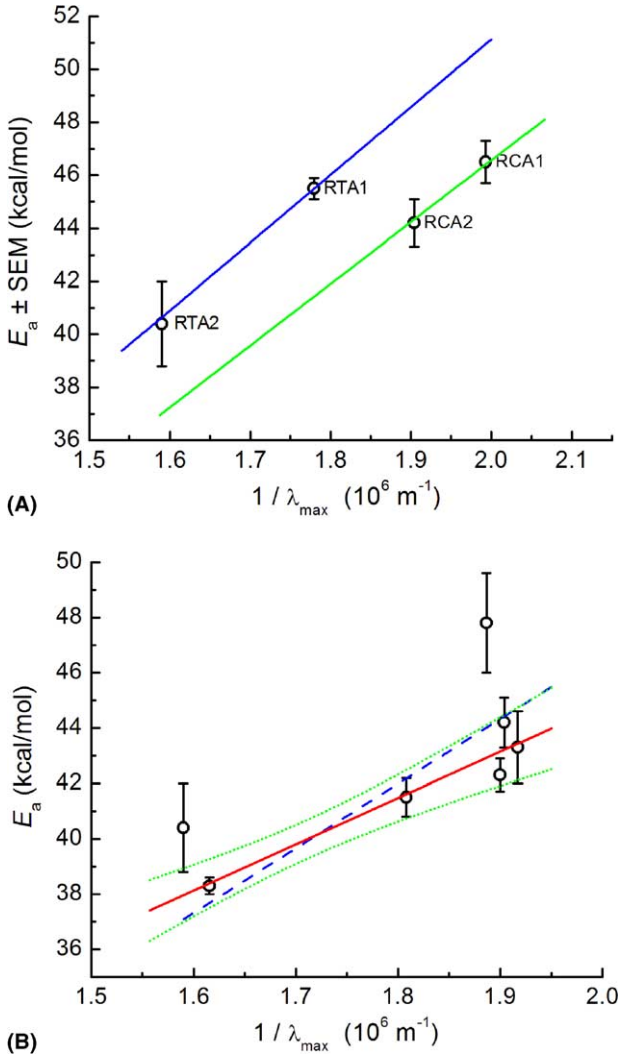

Fig. 3. The data from Fig. 2 regrouped to distinguish (A) the effect of a change from chromophore A1 and A2, (B) the effect of opsin differences between pigments with the same chromophore. (A) The two leftmost points plot the A2 (tadpole) and A1 (adult) version of the *Rana temporaria* L-cone pigment (RTA2 and RTA1). The blue line is the best SLB fit. The two rightmost points plot the A2 and A1 version of the rod pigment of adult *Rana catesbeiana* (RCA2 and RCA1). The green line is the best SLB fit. (B) All the A2 pigments from Fig. 2 provided with the best weighted linear regression fit (red line) and the best SLB fit (blue dashed line). The green dotted lines represent the narrowest confidence limits of the regression line that would encompass the SLB line. They delimit the 88% confidence interval. The origins of the A2 pigments are (from left to right, in order of increasing $1/\lambda_{max}$): (1) L-cones of *Rana temporaria* tadpoles, (2) L-cones *Carassius carassius*, (3) *Mysis relicta* (lake population), (4) *Mysis relicta* (sea population), (5) rods of *Carassius carassius*, (6) porphyropsin rods of *Rana catesbeiana*, (7) rods of *Xenopus laevis*.

about 10%. This range of variation would be enough, e.g., to raise the activation energy of the frog L-cone A1 pigment (see Fig. 2) from a hypothetical value on the SLB line to that experimentally observed. Also, it could of course easily account for the deviation of the experimental slope of A2 pigments from the SLB relation (Fig. 3B).

4. Discussion

The collected data suggest that evolution has had considerable but not unlimited freedom to tune E_a and λ_{max} independently. As shown earlier, the same E_a may be connected with quite different λ_{max}, the same λ_{max} with quite different E_a (Koskelainen et al., 2000). Here we have for the first time derived a quantitative expression for their (statistical) interdependence. It approximates the SLB relation rather closely. The impression is that this simple physical relation constitutes an underlying main component, on which the specific structural solutions of individual opsins superimpose additional variation.

In the case of a chromophore change, the data follow the SLB relation very accurately, and the situation may indeed not be much more complex than envisaged by Stiles (1948) and Barlow (1957). The extra double bond in 3,4-dehydroretinal (A2) compared with retinal (A1) lowers the energy gap from the ground state to the first electronically excited state (see e.g. Milder, 1991), and this red-shifts the absorbance spectrum of the free chromophore in ethanol from ≈381 to 401 nm (see e.g. Bridges, 1972). The Schiff-base binding of the chromophore to the opsin increases λ_{max} for both A1 and A2, but it seems unlikely that interaction with *the same* opsin should affect the $E_a - \lambda_{max}$-*relation* very differently for the two chromophores.

Modification by changing amino acid residues in the opsin offers an enormously larger number of degrees of freedom. The variation of E_a around the main trend may be taken as a rough measure of the leeway in molecular $E_a - \lambda_{max}$-coupling. If it is accepted that E_a correlates negatively with thermal noise (Barlow, 1957; see further below), it is an almost inescapable thought that, whenever thermal noise is liable to limit visual sensitivity, evolution should use the freedom it has to set E_a as high as possible consistent with a given λ_{max}. This would especially concern long-wavelength-sensitive pigments. From this point of view it is not really surprising that the drop in E_a with increasing λ_{max} is somewhat less steep than the SLB relation.

All the measurements on which the present E_a values are based involved light excitation in conjunction with temperature changes (cf. Lythgoe & Quilliam, 1938; St. George, 1952). Therefore E_a and λ_{max} both reflect the properties of the photoexcitation pathway and the conclusions are not as such sensitive to the question of differing molecular pathways for photic and thermal activation (Barlow, Birge, Kaplan, & Tallent, 1993; Firsov et al., 2002). We wish to point out, however, that there are strong a priori reasons to think that the activation energies for the two pathways should not in fact differ much. After photon absorption, the all-*trans* bleaching intermediate bathorhodopsin downhill from the peak of the ground-state energy surface for

chromophore isomerisation, is formed within 200 fs (Schoenlein, Peteanu, Mathies, & Shank, 1991; Wang, Schoenlein, Peteanu, Mathies, & Shank, 1994). This extremely fast photochemical reaction requires that the excited-state and ground-state surfaces be close, hence a fairly strict coupling between the ground-state energy barrier and the photoactivation energy E_a (see e.g. Mathies, 1999). The generally held notion that the two are very different appears to be based on a too simplistic analysis of experimental data (Baylor et al., 1980; Matthews, 1984). In a separate article (Ala-Laurila et al., 2004), we develop this point in detail and use the relation between the photoactivation energy E_a and λ_{max} given by Eq. (1) as an element in modelling the relation between λ_{max} and pigment-derived thermal noise in photoreceptor cells.

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