Spectral and thermal properties of amphibian visual pigments related to molecular structure

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II Fyhrquist, N., Govardovskii, V.I., Leibrock, C., Reuter, T. (1998). Rod pigment and rod noise in the European toad *Bufo bufo*. *Vision Research 38:483–486.*

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IV Koskelainen, A., Ala-Laurila, P., Fyhrquist, N. and Donner, K. (1999). Measurement of the thermal contribution to sensitivity of photoreceptors. *Nature*, in press.

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List of abbreviations

cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
E_a	activation energy
ERG	electroretinogram
GDP	guanosine diphosphate
GTP	guanosine triphosphate
λ_{max}	wavelength of peak absorbance
mRNA	messenger RNA
MSP	microspectrophotometry
PDE	phosphodiesterase
SNR	signal-to-noise ratio

Three-letter abbreviation	Amino acid
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

Abstract

Absorbance spectra, energy barriers for activation, and rates of thermal isomerization-like "dark" events of visual pigments were measured in retinal rods and cones. Rod visual pigments of anuran amphibians that had been characterized for at least two of these functional properties were sequenced (*Rana temporaria*, *Bufo bufo*, *B. marinus*) and compared (additionally *Rana catesbeiana*, *R. pipiens* and *Xenopus laevis*). The purpose was to clarify the relation between the three functional properties studied, and to identify amino acid substitutions in the opsins that might underlie differences in the thermal properties of spectrally similar pigments.

Spectra were measured by microspectrophotometry in single photoreceptors and electrophysiologically as sensitivity spectra of the mass light response from the respective photoreceptor type in the intact retina. "Dark" event rates were measured in *Bufo bufo* rods by the suction pipette technique and collected from the literature for rods of other species. Activation energies were estimated from the effect of temperature on spectral sensitivity at very long wavelengths. Sequencing was done by dideoxy chain termination.

Microspectrophotometrical study of 39 visual pigments from fishes, amphibians and reptiles, with maximum absorbances distributed over a wide spectral range from UV to red, showed that all vertebrate absorbance spectra can be described by either of two universal templates (one for chromophore A1 and one for A2) with the wavelength of peak absorbance (λ_{max}) as sole variable. Thus, λ_{max} can be used to completely characterize the absorbance spectrum of a pigment.

The measurements of activation energies (E_a) in spectrally different (frog rods vs. red cones) and spectrally similar (frog vs. toad rods) pigments showed that there exists no necessary connection between λ_{max} and E_a. Moreover, comparison of E_a with rates of "dark" events showed no correlation between these properties in A1 pigments. Similar measurements in A2 pigments, however, indicated that chromophore substitution from A1 to A2 is associated with decreased E_a and increased rates of "dark" events, besides a spectral red shift.

The main conclusion from the study of E_a, λ_{max}, and "dark" event rates is that no necessary connection exists between the three. This falsifies a widely held hypothesis, according to which both λ_{max} and "dark" event rates basically reflect the energy barrier for activation of the pigment molecule. On that hypothesis, λ_{max} would be inversely proportional to E_a, and the rate of "dark" events would necessarily increase with decreasing E_a. The conclusion also implies that each of the properties may be tuned independently by structural changes in the visual pigment molecule. The present study of rhodopsin sequences represents the first attempt to identify amino acids that may specifically affect thermal stability or activation energy without influencing the absorbance spectrum. The analysis singled out 4 candidate amino acids for further study.

1. Introduction

The molecules responsible for light detection – visual pigments – are of ancient origin. The same molecular structure – a protein (opsin) that traverses the cell membrane seven times, incorporating a light-capturing chromophore – is found in animals, plants and bacteria (Nilsson, 1996). By contrast, different photoreceptor cells and eyes have evolved several times independently to suit the needs of different life forms in their particular environments (Salvini-Plawen & Mayr, 1977). Regardless of the particular solutions, however, the physical character of light and the properties of the pigment molecules that catch the light always set an ultimate limit to light sensitivity (Barlow, 1982a & b).

According to a highly influential idea originated half a century ago (Stiles, 1948; de Vries, 1948; Lewis, 1955; Barlow, 1957), three central functional properties of visual pigments – absorbance spectra, the energy barrier of activation, and the probability of "spontaneous" activation by thermal energy alone – are necessarily interconnected: high sensitivity to long wavelengths (low-energy photons) would entail a low energy barrier of activation, and therefore a high probability for purely thermal activation. Thermal activation would produce spontaneous events identical to those due to photon absorptions, constituting an irreducible light-like noise that would limit the sensitivity of light detection (Barlow, 1956). It has been experimentally shown that rods do produce electrical events indistinguishable from the responses to single photons even in absolute darkness (Baylor *et al*., 1980).

In this thesis, the relation between spectral properties, activation energy and "dark" event rates of visual pigments were studied in photoreceptors of frogs and toads. The main conclusion is that there exists no necessary connection between the three. Thus each of them may be tuned independently by structural changes in the visual pigment molecule. In a first attempt to identify amino acids that may specifically affect thermal stability or activation energy, visual pigments with similar spectral absorbance but different rates of thermal activation were sequenced and compared. The functional effects of particular amino acids may be universal and applicable to a wide range of proteins and enzymes, with possible clinical and industrial importance.

2. Review of literature

2.1. Evolutionary perspectives

2.1.1. Photoreceptor evolution

A characteristic common to developed photoreceptor cells is the great increase in membrane surface for deposition of visual pigments. Generally, photoreceptors are divided into two major groups: those that evolved from cilia, and those that did not. Vertebrate rods and cones are of the ciliary type, whereas both groups occur in invertebrates. Accordingly, there is no strict phyletic explanation to the types of photoreceptors in different animals (Goldsmith, 1990). The great diversity in photoreceptor morphology has suggested at least 40 to possibly 65 evolutionary lines (Salvini-Plawen & Mayr, 1977). However, the occurrence of nearly identical visual pigments in eye structures that are apparently not homologous (Goldsmith, 1990; Fernald 1997), raises the intriguing question as to what other characters are monophyletic. Homologous master control genes with ancient origin trigger the morphogenesis of polyphyletic eyes (Halder *et al*., 1995; Nilsson 1996; Oliver & Gruss 1997). Eyes have evolved independently, but a number of homologous elements are involved.

2.1.2. Opsin evolution and molecular properties

Stuctural studies have shown that all visual pigments are members of a large class of proteins that are assumed to derive from a common ancestor. This class includes bacteriorhodopsin, which is a proton pump rather than a visual pigment, and all β-adrenergic receptors. Members of this class share a common design: the protein part of the molecule,

called opsin in visual pigments, forms helices that traverse the membrane seven times.

The length of the opsin molecule varies (348–382 amino acid residues), and some parts of it are more strictly conserved than others. Phylogenetic trees constructed on the basis of amino acid differences between the proteins suggest rod opsins diverged from middle-wavelength-sensitive cone opsins sometime in the Mesozoic (Okano *et al*., 1992; Hisatomi *et al*., 1997, Das *et al*., 1999; Goldsmith 1990).

Studies involving site-directed mutagenesis, chromophore analogues, and other techniques, have helped to pin-point sites of functional importance in the rhodopsin molecule. Opsin binds to retinal via a protonated Schiff's base at lysine^{296}, and glutamate^{113} serves as the retinylidene Schiff's base counterion (Sakmar *et al*., 1989: Zhukovsky & Oprian, 1989). Replacement of glutamate^{113} for glutamine produces a massive shift of spectral sensitivity from 500 to 380 nm. In addition, several other amino acid residues influence absorbance characteristics as well (Table I, p. 18). However, their influences are not as large as that of glutamate^{113}.

Two cysteines at positions 110 and 187 form a disulfide bridge that is essential for the formation of the correct tertiary structure of rhodopsin (Karnik *et al*., 1988). Rhodopsin is covalently modified by the addition of N-linked oligosaccharides at asparagine^{2} and asparagine^{15}, and palmitoylation at cysteine^{322} and cysteine^{323} (Ovchinnikov et al., 1988). Histidines at positions 65, 152 and 211 influence the transition between photoproducts MI and MII (Weitz and Nathans, 1992), and there are numerous residues that influence rhodopsin catalytic activity rates, chromophore regeneration, and that interact closely with retinal by forming hydrogen bonds (Nakayama & Khorana, 1991). Cross-linking certain helices in rhodopsin through metal-ion-binding sites prevented activation of transducin, the next step in the transduction cascade (Sheikh *et al*., 1996). This along with results from studies involving disulfide cross-link (Cai *et al.*, 1999) and nitroxide side chain (Altenbach *et al.*, 1999) incorporations into the molecule indicate that in order to bind and activate transducin, the helices in opsin must move respective to one another. Once the protein-chromophore complex is in its active conformation, activity is quenched by phosphorylation of threonines and serines at the carboxy terminal, followed by the binding of arrestin to the active site (Wilden *et al*., 1982; Kühn *et al*. 1984). A crude projection map of the molecule that reveals the location and tilting of the seven helices with respect to one another is available (Schertler *et al.*, 1993; Unger *et al.*, 1997).

Defective disorders such as retinitis pigmentosa and night blindness arise from any of a number of mutations in the rhodopsin molecule, which render it incapable of folding properly, inserting into the membrane, and/or binding retinal (Gal *et al*., 1997).

2.1.3. The chromophore

In vertebrates, the most commonly used chromophore is retinal (A1). Some fish, amphibians and reptiles have a second kind of chromophore, 3-dehydroretinal (A2) (Dartnall & Lythgoe, 1965). In insects (Diptera, Lepidoptera, and several other orders) the chromophore has been identified as 3-hydroxyretinal (Vogt, 1989) and 4-hydroxyretinal based visual pigments have been found in a bioluminiscent squid (Matsui *et al*., 1988). Furthermore, in flies, a second chromophore, 3-hydroxyretinol, attaches to the opsin via hydrogen bonding. This chromophore acts as a sensitizing pigment. The sensitizer absorbs maximally in the near ultraviolet (~350 nm) and the visual pigment absorbs with a maximum at 500 nm, thereby producing a dual-peaked spectral sensitivity in fly photoreceptors (Vogt, 1989).

3-dehydroretinal differs from retinal by one extra C=C bond in the p-electron system of the molecule, and acts by red-shifting absorbance characteristics of the visual pigment molecule in a regular manner (Dartnall & Lythgoe, 1965). Rod visual pigments that bind A1 are referred to as rhodopsins, whereas those binding A2 are porphyropsins. Visual pigments based on 3-hydroxyretinal or 4-hydroxyretinal are referred to as xan-

thopsins, and they are far more polar than rhodopsins and porhpyropsins (Vogt, 1989).

The evolutionary significance of choice of chromophore in general is uncertain. However, a plausible reason for changing from A1 to A2 in fish and amphibians is to match spectral sensitivity to predominantly longer wavelengths in fresh water (Lythgoe, 1972). In frogs of the genus *Rana*, the larval stage, the tadpole, develops in fresh water ponds. Tadpole retinas contain mainly A2, and thereby all visual pigments, in both cones and rods, are accordingly red shifted. During metamorphosis and the subsequent transition to a terrestrial lifestyle, A2 is gradually replaced by A1 (Wald, 1947; Liebman & Entine, 1968; Reuter, 1969). In the adult form there are no traces of A2 in the retina in most *Rana* species, except for in the American bullfrog, *Rana catesbeiana*, which is known for unusually aquatic habits. In this frog, the dorsal rim of the adult retina contains A2, whereas the ventral retina contains A1. It has been suggested that the animal would look down into the reddish fresh water with the dorsal part of its retina, whereas the ventral part would look up into the bright blue air (Reuter *et al.*, 1971). In the clawed frog, *Xenopus laevis*, tadpoles have a mixture of A1 and A2 that is replaced mainly by A2 in the adult retina (Crescitelli, 1973).

Vitamin A2 has, surprisingly enough, not been found in toads of the genus *Bufo*, even though the animals employ similar developmental patterns as frogs do (Peskin, 1957; Partridge *et al*., 1992).

2.1.4. Cones and rods

In vertebrate retinas with dual vision there are two classes of photoreceptors: cones that are used in bright light conditions during the day and rods that are used in dim light. They both consist of an inner segment where metabolic processes take place, and an outer segment that is packed with visual pigment molecules. Vision is initiated in the outer segment (Dowling, 1987; Rodieck, 1998).

Generally, rods have larger outer segments than cones do (Barlow & Mollon, 1982). Furthermore, in cones the photosensitive membrane is folded and continuous with the cell membrane, whereas in rods it is predominantly internalised as discs within the outer segment (Rodieck, 1998). However, in various animals there are rod-like cones, and cone-like rods (Goldsmith, 1990). However, cones and rods can also be discriminated by their array of specific isoforms of phototransduction proteins (Vinnikov, 1982) and by their physiological properties (Rodieck, 1998).

High light sensitivity in the rod system is due to large receptor cells, large collecting areas, and long summation times, which allow extensive collection of photons (Barlow & Mollon, 1982). Summation areas in the cone system are generally smaller, and responses are faster, which reduces sensitivity. Furthermore, cones amplify light signals to a much lesser extent than rods do: cone response amplitudes to single photons are only 5% of that of rods (Barlow & Mollon, 1982; Rodieck, 1998). However, the fundamental reason why the cone system cannot "see" single photons is that the small events are buried in noise produced by biological activity in the cone cell. There is some evidence that this noise could largely originate in thermal activation of the visual pigment, which would suggest that the cone pigment is more than 10000 times less stable than that of rods (Lamb & Simon, 1977; Schnapf et al., 1990; Donner, 1992).

The interaction between the opsin and the chromophore in rod visual pigments differs from that in cone visual pigments, producing slower regeneration rates, slower dark adaptation (Kefalov *et al*., 1999), and presumably less noise. The separation of the disks that contain most of the pigment from the outer cell membrane in rods may slow down recycling of chromophores between the photoreceptor and the pigment epithelium, thereby influencing the course of dark adaptation. In cones, photosensitive membranes are contiguous with the cell membrane, and thereby have direct access to the pigment epithelium (Barlow, 1982a; Barlow & Mollon, 1982; Kefalov et al., 1999).

According to visual pigment phylogenetics, cones probably preceded rods. It can

be argued that the generation of rod vision, a highly light-sensitive and thermally stable system, is more demanding and therefore would have evolved later than cone vision, and perhaps even from cone vision. Indeed, rods appear to pass through a stage of cone-like morphology while developing ontogenetically (Vinnikov, 1982).

2.2. The noise concept

The word noise makes most of us think of one sound that interferes with another particular sound that one is trying to perceive, for instance, the rumbling engines of an aircraft that drowns the sound of a human voice. This is the exact meaning of the word, which has also later been applied in situations involving physical stimuli other than sound.

A faint light signal may drown in the arbitrarily fluctuating distribution of background light. A photon is the smallest possible, indivisible unit of light, with an energy content E proportional to frequency n. Light intensity is equal to the number of photons per area and time unit. Its spectral composition refers to the distribution of photon energies (thus wavelengths).

Due to unavoidable variations in the number of photons emitted by the light source, any signal carried by light will be noisy, i.e. contain random variation. Let N equal the number of photons absorbed by a noiseless detector. Assuming that photons are Poisson distributed, the noise N^* is equal to the square root of the mean number of photons N:

$$N^* = \sqrt{N} \quad (1)$$

Consequently, in a smaller signal, the relative variation is larger.

The well-known concept signal-to-noise ratio (SNR) is defined as the signal mean divided by the standard deviation, and bears the same relation to the number of errors in all communication systems. Here, the standard deviation is equal to the square root of the mean, which gives

$$\mathrm{SNR} = N/N^{1/2} = N^{1/2} = \sqrt{N} \quad (2)$$

When the signal increases linearly, the noise component increases as the square root of the mean signal. The expression permits calculation of an upper limit to the reliability of flash responses produced by the physical stimuli.

However, other sources add to the noise component. Three additional components of photon or photon-like noise may arise from activation of visual pigment: first, activation due to a "background" flux of photons, second, activation by thermal energy in the absence of light, and third, activation by photoproducts following intense light bleaching, presumably by a back reaction of inactivated metarhodopsin II (Lamb, 1987). Noise is associated with the other transduction reactions as well, but elementary events initiated later in the chain undergo smaller amplification and do not appear as "photon-like". Thus the "continuous" dark noise (Baylor et al., 1980) is probably due to spontaneous activation of transducin (Lamb, 1987) and PDE (Rieke & Baylor, 1996). A further (smaller) component of noise probably arises from random opening and closing of cGMP-gated channels in the outer segment (Lamb, 1987).

The processes that follow thermal activation of a visual pigment are indistinguishable from that triggered by a photon: the two reactions produce identical electrical events in photoreceptors. On this view, the "dark light" or "inner background light" is the rate of thermally generated photon-like events, giving within a certain summation area and time a number of events D. This has the same effect on SNR as true background photons, reducing cell SNR to

$$\mathrm{SNR} = N/(N+D)^{1/2} \quad (3)$$

If the physical stimulus is not given in total darkness, randomly arriving photons from the background light reduces SNR further. If B is the number of background photons absorbed in one summation area and time,

$$\mathrm{SNR} = N/(N+D+B)^{1/2} \quad (4)$$

The general message of eqn. (4) is that any photon-like noise will necessarily impair light detection. For example, increasing B or D will necessarily increase the "signal" N needed for detection with a constant reliability (fixed error rate) (Barlow, 1982a; Aho *et al*., 1987). Thus, any means of decreasing the frequency of thermal events in the photoreceptor (thus D) will improve performance. The options for this meet a definite limit in the properties of photoreceptors and visual pigments.

2.3. Noise and rod vision

As suggested by the preceding section, vision is basically a statistical problem. At low light levels the small number of photons and their unavoidable stochasticity limits the amount of information received from the environment. The information actually available to the organism is further decreased by all sorts of intrinsic biological variation, noise.

The larger the sample, the richer the information, as is true for any statistical sample. One way of increasing the number of photons sampled is by collecting them over large areas and long times. This, however, correspondingly degrades spatial and temporal resolution (Barlow, 1982b). In rod vision, where high light sensitivity is important, fine details and colours are second in priority. Here, scarcity of photons is the challenge. The rod system optimizes photon capture by means of large receptor cells, long summation times, and large summation areas (the last being a property of the retinal wiring).

Large receptors packed with a large amount of visual pigment molecules catch photons efficiently. However, large numbers of visual pigment molecules with a non-zero (although extremely low) probability of thermal activation in one single cell will elevate the level of background noise. This is harmful to a system designed to detect single photons.

Nocturnal animals enhance photon capture by a number of optical solutions, e.g., large eyes with wide pupils (Warrant, 1999) and a reflecting tapetum lining the backs of the eyes, which makes light incident on the retina pass through the receptors twice (Rodieck, 1998). However, the size of the eye is limited by head size and/or the animal's energy budget, and the benefit of a tapetum is limited by resolution problems. For cold-blooded toads the photon-sample-size problem is alleviated by the slow life style (and slow prey), giving modest needs of temporal resolution. In addition, biological sources of noise are depressed at the low body temperature. Indeed toads and frogs are remarkably sensitive to light in comparison to humans. Were human and frog retinas exposed to the same temperature, their visual sensitivities would be stunningly similar. Toad behavioural thresholds are predicted by a straight line in a log diagram, falling with a slope of -1.26 ± 0.03 log units per 10 °C (Aho *et al*., 1993b). Human rod thresholds at 37 °C fit that trend relatively well – performance being slightly better than the expected (Fig. 1). The change in sensitivity by one order of magnitude in the frog retinas with a temperature rise from 10 °C to 20 °C is accounted for by at least two factors in roughly equal proportions: decreased temporal summation, and increased retinal threshold (flash sensitivity). Flash detection apparently drops due to increased retinal noise at the higher temperature (Copenhagen *et al*., 1987; Aho *et al*., 1988, 1993b).

2.4. Functional properties of visual pigments

Visual performance in animals eventually depends on information delivered by the retinal cells. As photon energy is converted into chemical signals in the photoreceptors, the retina creates an electrical image of the outside world. The visual pigments in the photoreceptors constitute the very first step in this chain of reactions. Therefore, they should catch photons as efficiently and reliably as possible.

At least three functional properties of visual pigments influence the efficiency and reliability of photoreceptors as light detectors.

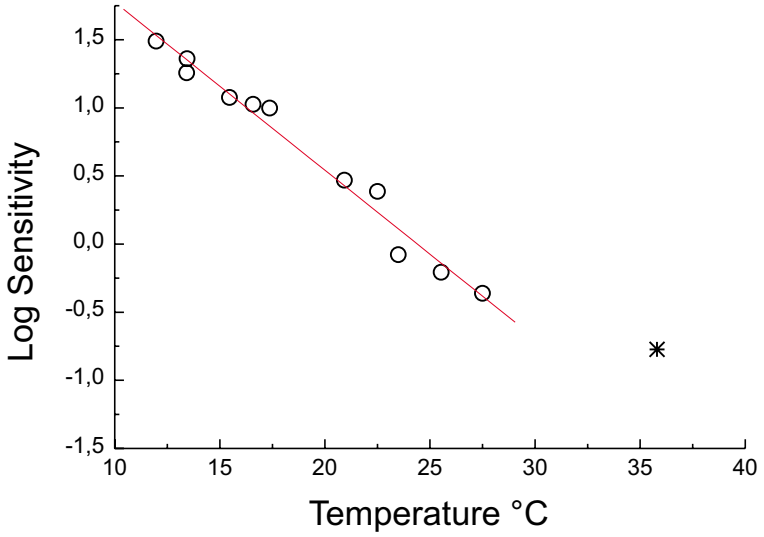

Figure 1. Dark adapted scotopic sensitivity in two frog species (○) and humans (∗) as a function of temperature. Ordinate, log sensitivity = – log threshold (threshold intensity at cornea (quanta $mm^{-2}s^{-1}$). Modified from Aho *et al.* (1993b) and Donner (1998).

One is spectral sensitivity, another is quantum efficiency, and a third is the tendency to generate thermal noise events in the absence of light. Visual sensitivity depends on these three properties, which in turn are determined by visual pigment structure. Visual pigments consist of two parts, a protein and its prosthetic group, the chromophore. Both of these influence function.

2.4.1. The art of catching light

Light exists in discrete energy packages called photons. The energy content of light is therefore quantized, and the size of the quanta depends on the wavelength of the light. Likewise, the photon detectors are excited by discrete packets of energy. Photons that are rich enough in energy excite visual pigments with given probabilities. No physical explanation has been obtained to explain the particular shape of the absorbance curve of visual pigments, except for the very long wave part of the spectrum, where each photon has less energy than the minimum needed for pigment activation (Stiles, 1948).

Experiments have shown that in the deep red, thermal energy may contribute to visual excitation (de Vries, 1948; Denton & Pirenne, 1954; Lewis, 1955; Srebro, 1966; Lamb, 1984). Visual pigment molecules may occupy a large number of vibrational energy levels, being distributed on these according to the Boltzmann law. In a large population of visual pigments, at least a small number contain enough energy in appropriate vibrational modes to supplement photon energy in producing activation. This fraction of molecules decreases as the energy contributed by the photon decreases, which would explain why sensitivity declines exponentially in the "red" end on a frequency scale (Stiles, 1948).

2.4.2. The initiation of rod responses

The reaction that follows the activation of a visual pigment by either light or thermal energy is rather complicated, and involves several steps of biochemical processes in the receptor, before a signal is transmitted to the following layer of cells. However, the phototransduction cascade has been elucidated more or less to its full extent, even though some regulatory processes and quantitative aspects on shutdown of the process are still somewhat unclear.

As light is absorbed by the chromophore, it isomerises from the chemically strained 11-cis configuration to all trans retinal (Hubbard & Wald, 1952), which activates the protein part of the molecule. Activated opsin undergoes structural changes that involve tilting and sliding of the helices respective to one another (Farrens *et al*., 1996), which enables

binding and activation of a G-protein, transducin. Transducin converts from an inactive state with GDP bound to an active state with GTP bound, which in turn activates PDE. Activated PDE cleaves cGMP that acts as a cation channel gatekeeper in the outer segment of photoreceptors. The reduction in cGMP concentration closes cation channels (Fesenko *et al*., 1985).

In the dark, the membrane is partially depolarized by a steady inflow of sodium through the cation channels in the outer segment. The current loop is closed by diffusion of potassium out of the inner segment through voltage dependent channels. Sodium/potassium ATPases in the inner segment maintain the proper ionic milieu inside the cell. As cation channels in the outer segment close as a result of photoactivation, the photoreceptor hyperpolarizes, and the signal is delivered to the following layer of cells through a decrease in glutamate release at the synapse. In the dark, glutamate is continuously released (Trifonov, 1968; Nunn & Baylor, 1982; Schnapf & Baylor, 1987; Stryer, 1987; Dowling, 1987; Ayoub & Copenhagen, 1991; Hargrave & McDowell, 1992; Rodieck, 1998).

Shutdown of rhodopsin activity is initiated by phosphorylation of rhodopsin by rhodopsin kinase. Phosphorylation enables the binding of arrestin to the active site of rhodopsin, which prevents further binding and activation of transducin molecules. The transduction cascade processes are regulated by negative feedback mediated by calcium ions. Ca^{2+} regulates the synthesis of cGMP by guanylate cyclase, influences phosphodiesterase and rhodopsin kinase activities, and reduces the affinity of cation channels for cGMP through calmodulin. As cation channels close, Ca^{2+} levels fall. The drop in Ca^{2+} restores the receptor current (Hodgkin *et al.*, 1985; Hargrave & McDowell, 1992; Baylor, 1996; Koutalos & Yau, 1996; Rodieck, 1998).

2.4.3. Reproducibility of rod photoresponses

Hecht, Shlaer and Pirenne (1942) suggested that the absorption of a single photon may activate a rod photoreceptor. This conclusion was drawn from psychophysical experiments: the number of photons required for threshold performance was so low that it was highly unlikely that any rod absorbed more than one photon.

With dim flashes, rod responses are quantal in nature: their amplitudes are proportional to the total number of photons absorbed. Due to the stochastic nature of light and irregular absorption of photons, the responses to dim light flashes of equal intensity vary arbitrarily. In addition, some photons absorbed by rhodopsins fail to elicit a response in the photoreceptor. The term quantum efficiency describes the ratio of successfully excited visual pigment molecules to the number of caught photons. In many visual pigments, quantum efficiency is about 0.67 (Dartnall, 1972), which means one molecule initiates activation of the transduction cascade per 1.5 quanta absorbed. The remaining 33% of photons have no known influence on the molecule, and are probably degraded into heat. Whether the 33% of photons that do not bleach rhodopsins, contribute to vision in another way, is unknown (Rushton, 1972). Given no attention in this thesis, quantum efficiency in visual pigments would deserve more investigations in the future.

Once rhodopsin has been activated, the size and shape of the rod response are remarkably constant (Baylor *et al*., 1979b, Schnapf, 1983, Rieke & Baylor, 1998), suggesting elegant control of the transduction cascade. High reproducibility of rod responses allows photon counting and timing with high precision.

Currently, activation of the transduction cascade is quantitatively well understood and mathematical models and simulations of molecular interactions coincide well with measurements done on a macroscopic level. The shape of the rising phase of the rod responses depends on several parameters: rhodopsin activation kinetics, linear gain in numbers of molecules of activated transducin and phosphodiesterase, co-operative interactions at the level of cation channels, and cation channel conduction dynamics (Lamb, 1996). However, the course of inactivation is still a

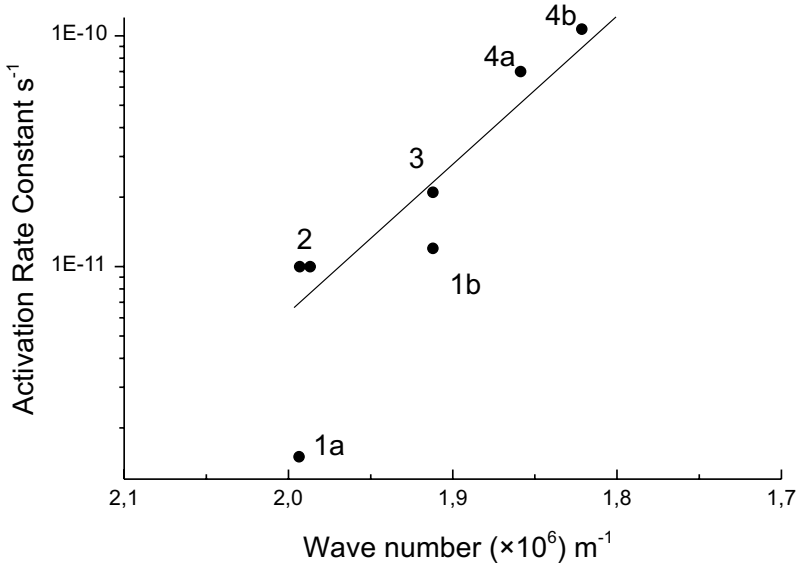

Figure 2. Thermal events per molecule (s^{-1}) in visual pigments as a function of λ_{max}. References as follows: 1a & 1b: Donner *et al.*, 1990; 2: Baylor *et al.*, 1980 and Paper II; 3: Donner *et al.*, 1997; 4a & 4b: Firsov & Govardovskii, 1990.

matter of controversy. Some regard it as a rigorously controlled multi-step process (Rieke & Baylor, 1998), others again as a stochastic one-step termination event (Whitlock & Lamb, 1999).

2.4.4. The generation of thermal events in rods

Photoreceptors are noisy (Baylor *et al.*, 1980; Matthews, 1984; Donner *et al.*, 1990; Firsov & Govardovskii, 1990; paper II), even though the visual pigment, rhodopsin, appears to be a very stable molecule with a half-life of at least 420 years (Schnapf & Baylor, 1987). However, since individual rods can contain over a billion rhodopsin molecules, thermal events occur with a finite frequency.

If thermal activations follow the same molecular route as photon-induced activations, the tendency to generate thermal dark events should depend on the energy barrier of activation of the pigment. The lower it is, the more frequent thermal events would be. Temperature influences the distribution of visual pigments molecules on thermal energy levels: an addition of heat shifts the distribution towards higher energies. This would increase the number of molecules that momentarily exceed the energy barrier for activation. Thus, thermal reactions would become more frequent as temperature rises (Stiles, 1948; Barlow, 1957).

In 1979 photoreceptor membrane currents were measured for the first time using the suction electrode technique (Baylor *et al.*, 1979a & 1979b, Yau *et al.*, 1979). It was noted that membrane current fluctuations identical to those elicited by photon absorption occasionally occurred in complete darkness (Baylor *et al.*, 1980). The rate of such "dark events" in toad red rods was 0.02 rod^{-1} sec^{-1} at room temperature, with Arrhenius-type temperature-dependence suggesting an activation energy of 22 kcal mol^{-1}.

It was suggested that the origin of dark events could be thermal activation of visual pigment molecules. Since they were indistinguishable in shape and duration from those elicited by real photoisomerisations, they must originate at the very beginning of the transduction cascade.

2.4.5. Spectral sensitivity and dark noise

The absorbance spectrum of a visual pigment (or sensitivity spectrum of a photoreceptor), has a unique maximum in the "visible" wavelength range; the wavelength of peak absorbance or sensitivity is denoted λ_{max}. The activation energy (E_a) is the minimum amount of energy required for excitation of a visual pig-

ment to activate the visual transduction cascade. It has been suggested that sensitivity to longer wavelengths, hence low energy photons, would imply a lower energy barrier of excitation in the visual pigment (Stiles, 1948; de Vries, 1949; Barlow, 1957). According to this theory, E_a would simply be inversely proportional to λ_{max}.

Dark noise in photoreceptors would depend on E_a, and thereby also on λ_{max}. Dark noise would be large in visual pigments with low E_a, and hence high λ_{max} (Barlow, 1957). Consequently, λ_{max}, E_a and dark noise, would constitute an inseparable "triad" of functional parameters, interconnected by physical necessity.

Indeed it has been shown that two different chromophores that occur naturally in frog rods, produce shifts not only in spectral sensitivity, but also in photoreceptor noise in the expected manner. Replacement of A1 with A2 induces red shifts (Dartnall & Lythgoe, 1965; Reuter *et al*., 1971) as well as higher rates of thermal activation (Donner *et al*., 1990) in rod cells (Fig. 2). The change in thermal stability is expected from the extra double bond in the p-electron system of A2 (cf. Williams & Milby, 1968). According to current views, increased rates of thermal activation of visual pigment molecules significantly degrades visual sensitivity (Copenhagen *et al*., 1987; Aho *et al*., 1987, 1988, 1993b).

2.5. Spectral sensitivity of visual pigments

2.5.1. The clustering of rod spectral sensitivity

Radiation below 300 nm is highly scattered by the atmosphere and has enough energy to break covalent chemical bonds. At wavelengths beyond 2 mm, self-radiation increases significantly, which again makes that part of the spectrum less useful for obtaining information (Dusenbery, 1992). The visible part of the spectrum lies inside these limits, photoreceptors being sensitive to wavelengths between 300 and 800 nm.

The discovery that the spectral sensitivity of visual pigments varied across species led to the speculation that evolution "designed" pigments to maximally absorb the wavelengths that predominate in the light environment particular to each species. This should be especially true for rod vision, where efficient photon catch is imperative.

Ocean water is most transparent to wavelengths in the "blue" part of the spectrum, and the spectrum of available light narrows down considerably at greater depths. In such an environment, one would expect blue-shifted rhodopsins (Bayliss *et al*., 1936; Clarke, 1936) and indeed the prediction proved to be correct in fish living at great depths (Denton and Warren, 1957; Munz, 1958; Fernandez, 1979).

In fresh water pools, the distribution of light is different from that in the ocean. Fresh water is relatively more transparent at longer wavelengths, and in waters strongly stained by yellow products of vegetable and phytoplankton decay, transparency is highest near the infrared (Muntz, 1978; Muntz & Mouat, 1984). In fresh-water fish, rhodopsins are indeed slightly red-shifted in comparison to those of terrestrial animals and shallow-water fish. However, for an optimal match to the spectrum of available light, they are not red sensitive enough.

The λ_{max} of vertebrate rods cluster around 500 nm for unknown reasons. One hypothesis suggests the narrow range of peak sensitivities (480–515, if rhodopsins based on 3-dehydroretinal are omitted) may be baggage of several other functional adaptations of rod vision that are perhaps not totally independent of one another (Goldsmith, 1990). As rods take over from cones during dark adaptation, overall spectral sensitivity shifts towards shorter wavelengths. This universal phenomenon, the 'Purkinje shift', cannot be explained in terms of sensitivity, since the ambient light at night is richer in the long wavelengths than daylight. Barlow (1956 &1957) suggested that there is an opposite pressure against long wavelength sensitivity, consisting of increased photoreceptor noise as an inherent property of visual pigments with maxi-

mal sensitivity to low energy photons. The need to reduce retinal noise to maintain reasonable signal/noise ratios, would force spectral sensitivity towards blue in rods. Noisy signals would be a smaller disadvantage in cone vision, as they operate at higher light intensities than rods.

2.5.2 Spectral tuning in visual pigments

Spectral tuning in visual pigments is accomplished by two means: either by switching chromophores which takes place on a physiological time scale, or by amino acid changes in the opsin molecule on an evolutionary time scale. Switching from A1 to A2 shifts spectral sensitivity of visual pigments in a regular manner towards red (Dartnall & Lythgoe, 1965).

The absorption maximum of human rhodopsin is at 498 nm (Nathans, 1990), whereas that of the protonated retinal Schiff's base free in methanol solution is at 440 nm (Erickson & Blatz, 1968). The 60-nm shift in spectral sensitivity, called the "opsin shift" is presumably caused by interactions between the protonated chromophore and the protein. Kropf and Hubbard (1958) proposed that photoexcitation of rhodopsin leads to an increased delocalization of p-electrons: the positive charge localized primarily to the Schiff's base nitrogen, is more evenly distributed throughout the p-electron system of the chromophore in the photoexcited state. They proposed that any interactions between the chromophore and the protein that favour delocalization will stabilize the activated state and thereby produce a red shift, and conversely, interactions that disfavour delocalization will produce a blue shift. Critically placed charged residues may have such effects (Nathans, 1990). Furthermore, amino acid side chains may produce sterical effects (Han *et al*., 1996), or bind ions (Wang *et al*., 1993), which produce spectral shifts of the visual pigment. Long wavelength sensitivity in cones appears to depend on the availability of chloride. The spectral sensitivity of chicken iodopsin, which absorbs maximally at 562 nm, was shifted by 50 nm towards shorter wavelengths (λ_{max} 512 nm) in chloride depleted solution (Shichida *et al.*, 1990). Wang et al. (1993) have shown that human red/green cone sensitivity depends on binding chloride ions as well.

Indeed, as soon as it became possible to use site-directed mutagenesis as a powerful tool for testing the effects of one or more amino acids, it was shown that certain amino acid residues in opsin did produce spectral shifts (Zhukovsky and Oprian, 1989; Neitz et al., 1991). Most of them are located in the transmembrane part of the molecule (according to the model by Hargrave et. al (1983), and by Baldwin (1993)) and involve either a non-conserved substitution or loss or gain of a hydroxyl group (Nathans, 1990; Nakayama & Khorana, 1990; Chan *et al*., 1992; Merbs & Nathans, 1993; Asenjo *et al*., 1994). Amino acid residues that have been assigned spectral tuner properties are listed in Table I. Residue Glu^{113} shifts spectral sensitivity of rhodopsin by as much as 120 nm. It serves as counterion to the Schiff-base linkage between the opsin and the chromophore.

It should be noted that just a few of the substitutions that have been performed in order to test the point-charge model (put forward by Kropf & Hubbard, 1958; Honig *et al*., 1976) are found in nature (Yokoyama, 1995). However, a number of amino acid changes that have been found in nature by comparing the primary structures of spectrally different visual pigments, have proved to shift spectral sensitivity by means of experiments. A good example are the three types of cones in the human retina that contain visual pigments maximally sensitive to approximately 420, 530 and 558 (552 or 557) nm, the latter depending on which polymorphic gene one possesses (Dartnall et al., 1983; Merbs & Nathans, 1992). Their deduced amino acid sequences show 96% identity between the red and the green visual pigments, and 40% identity in all other pairwise comparisons. The highly conserved properties of visual pigments, such as isomerization, transducin activation and phosphorylation are most certainly reflected in the conservation of amino acids in functionally important parts of the protein. Conversely, differences in amino

Table I. Amino acid residues that have been assigned spectral tuner properties.

Location* /residue	Substitution	Direction	Magnitude	Source
83	Asp-Gly	red	1.5 nm	Nathans, 1990
	-Asn	blue	8.5 nm	Nathans, 1990
	Asp-Asn	blue		Hunt *et al*., 1996
	Asp-Asn	blue	3 nm	Fasick *et al*., 1998
86	Met-Glu	blue	5.5 nm	Nathans, 1990
/83b	Met-Leu	blue		Lin *et al*., 1998
90				
/87b	Gly-Ser	blue		Lin *et al*., 1998
110				
/116r/g	Ser-Tyr			Asenjo *et al*., 1994
113	Glu-Gln	blue	120 nm	Sakmar *et al*., 1989
117				
/114b	Ala-Gly	blue		Lin *et al*., 1998
121	Gly-Ser	blue		
	-Thr, -Val, -Ile, -Leu		1–23nm	Han *et al*., 1996
122	Glu-Gln	blue	18 nm	Sakmar *et al*., 1989, Zhukovsky & Oprian, 1989
	Glu-Asp	blue	23 nm	Sakmar *et al*., 1989
	Glu-Gln	blue		Nakayama & Khorana, 1991
	-Asp, -Ala			
	Glu-Gln	blue	17 nm	Nathans, 1990
	Glu-Ile	blue	2 nm	Nathans, 1990
/119b	Glu-Leu	blue		Lin *et al*., 1998
124				
/121b	Ala-Thr	blue		Lin *et al*., 1998
134	Glu-Leu	blue	1nm	Nathans, 1990
135	Arg-Leu	red	1nm	Nathans, 1990
164	Ala-Ser	red	4 nm	Chan *et al*., 1992
/180r/g	Ser-Ala	blue	5 nm	Asenjo *et al*., 1994, Neitz *et al*., 1991
211	His-Phe	blue	4.5 nm	Nathans, 1990
	His-Cys	blue	5 nm	Nathans, 1990
	His-Glu	red	35 nm	Weitz & Nathans, 1993
214				
/230r/g	Ile-Thr			Asenjo *et al*., 1994, Neitz *et al*., 1991
217				
/233r/g	Ala-Ser			Asenjo *et al*., 1994, Neitz *et al*., 1991
261	Phe-Tyr	red	6–10 nm	Merbs & Nathans, 1993
/277r/g	Tyr-Phe	blue	7 nm	Merbs & Nathans, 1993, Asenjo *et al*., 1994, Chan et a., 1992, Yokoyama *et al*., 1995, Hunt *et al*., 1996, Neitz *et al*., 1991
/270**	Phe-Ser	red	5 nm	Morris *et al*., 1993
265	Trp-Tyr	blue		Nakayama & Khorana, 1991
	-Phe, -Ala			
/262b	Trp-Tyr	blue		Lin *et al*., 1998
269	Ala-Thr	red	16 nm	Chan *et al*., 1992
/285r/g	Thr-Ala	blue	14 nm	Merbs & Nathans, 1993, Asenjo *et al*., 1994, Neitz et al., 1991
292	Ala-Ser	blue	10 nm	Nakayama & Khorana, 1990
	Ala-Glu	red	35 nm	Weitz & Nathans, 1993
	Ala-Ser	blue		Hunt *et al*., 1996
	Ala-Ser	blue	10 nm	Fasick *et al*., 1998
/289b	Ala-Ser	blue		Lin *et al*., 1998
293	Phe-Glu	red	9 nm	Weitz & Nathans, 1993
/309r/g	Tyr-Phe			Asenjo *et al*., 1994, Neitz *et al*., 1991
295				
/292b	Ala-Ser	blue		Lin *et al*., 1998
299	Ala-Glu	red	15 nm	Weitz & Nathans, 1993
	Ala-Ser	red	2 nm	Fasick et al., 1998
/296b	Ala-Cys	blue		Lin *et al*., 1998
300	Val-Glu	red	16 nm	Weitz & Nathans, 1993

* Location numbers according to the primary structure of rhodopsin, r = red, g = green, b = blue cone equivalent positions.
** squid rhodopsin

acid composition may reflect differences in spectral sensitivity between the corresponding visual pigments (Nathans *et al*., 1986; Nathans, 1990; Nathans, 1992).

A study that compared 8 primate genes coding for cone visual pigments with spectral sensitivities in the range from 530 to 562 nm suggested three amino acid substitutions in the opsin sequence account for the 30-nm difference that underlies human red-green colour vision (Neitz *et al*., 1991). This study, along with the confirmation of the precise effect of the particular amino acids (Merbs and Nathans, 1993; Asenjo *et al*., 1994) have provided a beginning to understanding the mechanisms of spectral tuning.

It has been suggested that the same tuning mechanisms could operate similarly in all visual pigments. Indeed similar or identical amino acid changes at equivalent positions in both cone and rod opsins have been designated spectral tuning properties (see Table I). However, Nathans (1990) noted certain charge alterations produce much smaller effects in rhodopsin than in cone visual pigments.

Makino *et al*. (1999) have shown that the chromophoric ring electrons play a role in spectral tuning in red and blue cones, however, not in red rods. Glu^{113} produces most of the red shift in rods by serving as a counterion to the protonated Schiff's base linkage by perturbing C12 of the chromophore (Shieh *et al*., 1997). Red cones could achieve part of their red shift through an increased separation between the counterion and protonated Schiff's base linkage (Blatz *et al*., 1972).

In blue cones and amphibian green rods the effect of the opsin shift is opposite to that in red cones and red rods: the blue sensitive pigments are hypsochromically shifted compared to their protonated chromophores in methanol solution. The opsin shift in blue cones arises from perturbations near the Schiff's base linkage, in addition to perturbations of the chromophoric ring (Lin *et al*., 1998, Koechendoerfer *et al*., 1999; Makino *et al*., 1999). The surprisingly high estimates of dark noise in toad green rods (Matthews, 1984) in comparison to toad red rods (Baylor *et al*., 1980), may indicate a spectral tuning mechanism different from that employed in red rods. Deprotonation of the Schiff's base linkage as a possible spectral tuning mechanism of blue sensitive visual pigments has however, been eliminated by resonance Raman evidence against it (Loppnow et al., 1989).

The cumulative effects of amino acid residues on spectral sensitivity in visual pigment molecules need to be explained by future experiments. Recently, Fasick et al. (1998) have determined the additive effects of D83N, A292S and A299S in mutants expressing all possible combinations of single, double and triple substitutions. However, not until an exact tertiary structure of rhodopsin is available, will it be possible to determine the effects of various amino acid side chains in detail. At present, there is a projection structure map on rhodopsin at the resolution of 6 Å (Schertler *et al*., 1993; Unger *et al*., 1997).

2.5.3. Spectral sensitivity and absorbance in visual pigments

2.5.3.1. Absorbance curve templates

The large size of the chromophore (20 carbons) in visual pigments, in close interaction with opsin, allows a large number of internal energy levels within the molecule. The levels of rotational and vibrational energy may be very close together, or continuous with one another, which makes continuous and homogenous absorption spectra possible (St George, 1952).

All visual pigments have absorption curves of basically similar shape (Dartnall, 1953) that vary in proportion to λ_{max} (Ebrey & Honig, 1977) and are broadened by unsaturated bonds in the chromophoric ring (Makino *et al*., 1999). In order to generate a universal template for visual pigment absorbance, several mathematical expressions that take into account modifications of the shape of the curve as a function of peak sensitivity have been constructed (Dartnall, 1953; Dawis 1981; Partridge & De Grip, 1991; Mansfield, 1985; Stavenga et al., 1993). Mansfield (1985) elegantly generated one single tem-

plate, instead of three separate templates for different regions of the spectrum, by plotting absorbance spectra against normalized frequency ν/ν_{max} (or λ_{max}/λ, as ν is proportional to $1/\lambda$). The expression proposed by Lamb (1995)(based on the Mansfield transformation)

$$S(x) = \{\exp a(A-x) + \exp b(B-x) + \exp c(C-x) + D\}^{-1} \quad (5)$$

with modifications from paper I, where S(x) is normalized sensitivity as a function of normalized frequency ν/ν_{max}, A, B,C, and D are position constants, and a, b and c give the slopes of the three exponentials, describes visual pigment absorption rather well over a wide range of λ_{max}. In Lamb's model the best fit is given by: A = 0.880, B = 0.924, C = 1.104 and D = 0.655, a = 70, b = 28.5 and c = –14.1.

The absorption curve describes the probability that a photon of a certain wavelength will excite the visual pigment molecule. Based on the "principle of univariance", according to which a photon elicits photoreceptor responses of the same uniform size irrespective of wavelength, photoreceptor spectral sensitivity is supposed to be proportional to visual pigment absorbance.

The parameters that determine the shape of the curve are purely hypothetical , i.e. they still lack an adequate physical explanation, except for possibly the long wavelength part of the spectrum, which will be discussed below.

2.5.3.2. Factors that may distort the absorbance spectrum

There are several factors that may distort the shape of the absorbance spectrum. Some of them are due to visual pigment properties, others to experimental artifacts. The former includes Kundt's rule, and effects of the ionic milieu and extracting agents (i.e. detergents).The latter includes self-screening, and absorbance by substances such as photoproducts, other pigments, parts of the pigment epithelium, and other impurities. A mixture of two or more opsins or chromophores in the same cell will likewise influence the shape of the absorbance curve.

In the *in vitro* preparation, the photopigments are extracted from the retina by a detergent, i.e. digitonin, with which they form micelles in the aqueous solution. Digitonin may have effects on the native conformation of visual pigments. Cone pigments may be especially sensitive since their retinal binding site is more exposed to the surface of the molecule (Okano *et al*., 1989). According to Kundt's rule, visual pigment absorbance in extracts would be displaced to longer wavelengths compared to absorbance in a^n *in situ* preparation. The effect would be purely physical, due to a more refractive medium of the visual cells (Bowmaker, 1972). In paper I there is a 1 nm hypsochromic shift of the rhodopsin absorbance curve *in vitro* compared to the *in situ* preparation of frog rods, which could be attributed to Kundt's rule.

In the *in situ* preparation, the retina is isolated from the underlying pigment epithelium, and either maintained intact, or gently torn into pieces to isolate photoreceptor outer segments. Bowmaker (1973) suggested the biochemical milieu may be different in isolated outer segments, compared to outer segments attached to the retina. In paper I both kinds are measured, and no differences were found.

As for experimental artefacts, the effect of bleaching visual pigments is one important factor. Photoproducts, mainly metarhodopsin II, which form upon illumination, absorb in the visible region (Meta II at 380 nm), with an absorbance extending beyond 500 nm. Therefore, the true rhodopsin peak in the computed difference spectrum (dark spectrum subtracted by a post-bleach spectrum to monitor the composition of absorbing substances) will be displaced toward longer wavelengths. The addition of hydroxylamine, which forms retinal oxime with photoproducts, and absorbs maximally at shorter wavelengths (363 nm, absorbance decay steeper than for Meta II), abolishes the displacement in rhodopsins with λ_{max} longer than 480 nm (Bowmaker, 1972; Victor Govardovksii, personal communication). In the MSP recordings of paper I, the effect of retinal oxime is negligible since it

is oriented along the rod axis, and the light beam is polarized across the rod axis.

Any other impurities in the sample will have distorting effects as well. The occurrence of two different opsins, or chromophores in the same cell requires that their proportions should be properly monitored by partial bleaching and superimposing templates that describe the absorbance of both visual pigments.

Transilluminating the intact retina may bring about self-screening, which flattens the absorbance curve, since a large fraction of visual pigment molecules look through a "coloured filter" of visual pigment molecules. The wavelength that is best caught, will thus be sharply attenuated (Rushton, 1972). Furthermore, the spaces between the cells may allow a significant amount of light leakage between the outer segments (Victor Govardovskii, personal communication).

Finally, when attempting to compare visual pigment absorbance *in vit*ro and *in situ* to psychophysically determined sensitivity, the distorting effects of optical factors in the eye should be kept in mind.

2.5.3.3. Activation energy and sensitivity at long wavelengths

For isomerization of the chromophore and initiation of the transduction cascade to occur, a minimum amount of energy (E_a) must be absorbed. According to the theory first proposed by Stiles in 1948, the energy needed to activate rhodopsin need not be wholly derived from light, but may be supplemented by heat. Low-energy photons ($hc/\lambda < E_a$) may therefore excite rhodopsin, provided that the molecule contains enough internal energy E to provide an appropriate supplement of energy, so that the barrier of activation E_a is attained or exceeded ($E + h \geq E_a$).

With increased temperature, the fraction of molecules at higher levels of thermal energy will be larger, increasing sensitivity to longer wavelengths.

In paper IV, E_a is determined from the sensitivity differences at a number of wavelengths on the longwave tail of photoreceptor sensitivity spectra. The sensitivity difference is translated into its photon-energy equivalent by means of the local slope of the log sensitivity spectrum plotted on a wave number scale:

$$hc/\lambda_a = E_a = hc/\lambda_i + hc/T \; [-\log S/(1/T)]_i/[\log S/(1/\lambda)]_i \quad (6)$$

Here, $[-\log S/(1/T)]_i = (\Delta\log S1 - \Delta\log S2)/(1/T2 - 1/T1)$ is the sensitivity difference at two different temperatures, $T = T2$, and $[\log S/(1/\lambda)]_i$ is the local slope of the $T2$-spectrum at λ_i.

The energy barrier of activation of rhodopsin molecules has been estimated from bleaching experiments in vitro (Lythgoe & Quilliam, 1938) and measured directly with a photocalorimeter (Cooper, 1979), giving values of 44 kcal mol^{-1} and 45–48 kcal mol^{-1}, respectively. This corresponds to wavelengths 610–650 nm, and it has been observed that in this region, rhodopsin spectra start falling exponentially as a function of frequency (Goodeve, 1936; deVries, 1948; St. George, 1952; Srebro, 1966,Lamb, 1995; paper IV).

If thermal energy would not contribute, visual sensitivity would drop precipitously to zero beyond the limiting wavelength $\lambda_a = hc/E_a$. Indeed the absorbance band of rhodopsin solutions at very low temperatures (below –100 °C) is curtailed in the long wave end (Broda & Godeeve, 1941; Yoshizawa & Wald, 1966). Thus thermal energy expands the spectrum of light available for vision. A second important functional consequence of the thermal-energy contribution is the tendency to generate thermal "dark" events.

2.6. Uncoupling the functional triad: λ_{max}, E_a and dark events

Since visual pigment absorbance coincides with visual sensitivity according to the principles of univariance, spectral shifts in visual pigments will have immediate consequences for the light sensitivity of the organism. Photon catch is best if the visual pigments are tuned to maximally absorb the most commonly occurring photons. However, it has been suggested that the benefit of shifting

spectral sensitivity to longer wavelengths is opposed by an increasing tendency to generate thermal events, which lower SNRs and thereby raises retinal threshold (Barlow, 1957). Indeed, in nature, there appears to exist a reluctance to push visual pigment sensitivity very far out in the long wave part of the spectrum (Lythgoe, 1988).

The increased frequency of dark events in photoreceptors exposed to higher temperatures (Baylor *et al*., 1980) is evidence for the thermal contribution to the generation of dark noise. However, the activation energy (E_a) estimated from that temperature-dependence was only about half of that determined for thermal bleaching of rhodopsin in vitro (Lythgoe & Quilliam, 1938). At the time of publication, the authors (Baylor *et al*., 1980) gave little attention to the apparent discrepancy. Thirteen years later Barlow *et al*. (1993) suggested that dark noise is generated by a different molecular route, starting from molecules where the Schiff-base linkage of the chromophore to the opsin is deprotonated, which would be associated with a lower energy barrier of excitation.

The universality of the idea of a necessary relation between λ_{max} and dark noise was challenged, as more measurements of pigment-related electrical noise in rods became available. Matthews (1984) recorded unexpectedly high dark event rates in blue sensitive green rods of amphibians, and Donner *et al*. (1990) unexpectedly silent rhodopsin rods in the bullfrog (*Rana catesbeiana*) in comparison to spectrally identical rods of the marine toad (Baylor *et al*., 1980). Paper IV goes one step further in uncoupling the triad E_a–λ_{max}– dark noise, finding no necessary relation between λ_{max} and E_a. Thus, it may be assumed that spectral sensitivity and thermal properties of visual pigments have been subject to independent natural selection.

3. Aims of the study

The general aim was to clarify the nature of the relation between spectral properties, activation energy and "dark" event rates of visual pigments and to begin to relate the thermal properties to molecular structure.

In paper I, visual pigment absorbance was thoroughly examined by microspectrophotometry in rods and cones covering a wide spectral range. The purpose was to establish whether all spectra can be described by a universal template with the wavelength of maximum absorbance λ_{max} as sole variable. Given that this is possible (separately for A1 and A2 pigments), the parameter λ_{max} can be used for fully characterising the spectral properties of a pigment.

In paper II, the red rods of the European toad (*Bufo bufo*) were spectrally characterised and rates of thermal activation in complete darkness were estimated in order to provide the missing piece of information between thermal events on a molecular level and behavioural threshold in the same species.

In paper III, the primary structures of rhodopsins of three toad and frog species with similar absorbance spectra but differing thermal activation rates were determined, analysed and compared. The purpose was to map mutations in the amino acid sequences that could specifically alter thermal stability without affecting the spectral properties.

In paper IV, activation energies of spectrally similar and spectrally different visual pigments, both A1- and A2-based ones, were determined in amphibian photoreceptors. The purpose was to test the idea of a necessary relationship between spectral sensitivity and activation energy.

4. Material and methods

4.1. Isolation and preparation of retinas

Frogs and toads were decapitated and double-pithed, and retinas isolated under deep red light. The different species that were used in the studies I–IV are listed in Table II. In paper III, the retinas were immediately deep-frozen in liquid nitrogen, and stored in –70 ° C, after which one retina at a time was used for isolating mRNA, and synthesizing first-strand cDNA from purified mRNA. In all other studies, the retinas were kept in frog ringer solution and either gently pulled into pieces in order to expose single rod outer segments (I and II), or kept intact for mass potential recordings (IV).

Table II. List of species of toads and frogs used in studies I–IV.

Species	Paper I	Paper II	Paper III	Paper IV
Bufo bufo	X	X	X	X
Bufo marinus	X	X	X	
Rana catesbeiana	X	X		
Rana pipiens	X	R*		
Rana temporaria	X	X	X	
Xenopus laevis	X	A*	X	

R* retinal cDNA library kindly provided by Dr Wolfgang Baehr
A* available for the purposes of the study

4.2 Methods and data analysis

For most details, the reader is referred to the Methods sections of the original papers.

4.2.1. Microspectrophotometry (I & II)

When working with visual pigments with λ_{max}>500 nm, all procedures following dark adaptation were carried out in infrared light, using an infrared viewer (Find-R-Scope, FJW Industries, III). Pieces of retina were mounted between two glass cover slips in Frog ringer solution containing 10% dextrane to prevent excess movement of the rod outer segments during the recordings, and the edges were sealed with vaseline.

The absorption of single outer segments was recorded in a single beam computer-controlled microspectrophotometer, with a halogen lamp as a light source and the different wavelengths obtained with a grating monochromator. The raw spectra were corrected for zero offset by fitting the position of a zeroline to the long-wavelength tail (650–750 nm) of the spectrum, where visual absorbance using this technique is virtually zero. 20–60 spectra were normalized to 1.0 at 502 nm, averaged, and fitted with rhodopsin templates proposed by Partridge and DeGrip (1991) for A1 visual pigments, and Bridges (1967) for A2-based visual pigments.

Chromophore mixtures (A1/A2) could be analysed to the stringency of 2% admixture of either A1 or A2. This was done by adding given proportions of A1 and A2 templates, with their λ_{max}-difference constrained by the Dartnall-Lythgoe (1965) relation.

4.2.2. Electrophysiology (II & IV)

4.2.2.1. Suction pipette recordings (II)

Pieces of chopped retina (100–200 µm across) were transferred to a chamber on the stage of a microscope equipped with an infrared light source and an image converter. In a continuous stream of moist oxygen the outer segment of a rod was drawn into the tip of a glass micropipette, connected by an Ag–AgCl electrode to a current-to-voltage converter. The output voltage was proportional to the membrane current flow across the part of the cell drawn into the pipette. Precautions were made to prevent stray light from falling onto the preparation when making dark recordings. The rate of discrete events was estimated by visual inspection. The experiments were performed by Cornelia Leibrock and Tom Reuter.

4.2.2.2. ERG mass potential recordings (IV)

Intact retinas were mounted in a specimen holder with the photoreceptor side illuminated and continuously perfused by Ringer,

containing 2 mM aspartate to block synaptic transmission. Spectral sensitivity was measured at two temperature intervals (5–8 °C referred to as cold, and 25–28 °C referred to as warm) using 29 interference filters covering the range 397–802 nm. For monitoring changes in the saturating response amplitude, intensity-response functions were measured at regular intervals at a reference wavelength throughout the experiments.

The log of sensitivity at each wavelength relative to that at a reference wavelength was determined. Values across experiments for each receptor type were averaged and the warm and cold temperature curves were carefully aligned. The differences between the cold and the warm spectra at long-wavelength points were converted to photon energy equivalents through equation (6) in section 2.5.3.3.

4.2.3. cDNA sequencing (III)

First-strand cDNA was amplified in polymerase chain reactions (PCR), using oligo dT primers against the alligator rhodopsin amino terminus and carboxy terminus regions, and an exact primer designed against a portion of *Rana catesbeiana* 5' untranslated region. Amplified products were either cleaned or gel-purified, cloned into plasmid vectors, and sequenced by the dideoxy chain termination method (Sanger *et al*., 1977).

The nucleotide sequences, translated into amino acid sequences, were aligned using the multiple alignment program Clustal W (Thompson *et al*., 1994). Phylogenetic analyses were performed using PAUP version 3.1 (Swofford, 1993), using the branch and bound search feature.

5. Results

5.1. Modification of the Lamb template to fit visual pigments with extreme values of λ_{max} (I)

Are absorbance spectra generated from visual pigment extracts comparable with absorbance *in situ* ? Is absorbance proportional to spectral sensitivity? Are the templates that exist today applicable over a broad range of λ_{max} ?

In order to answer the above questions, first, the absorbance spectra of red rods of six amphibian species (listed in Table II) were measured *in situ* and *in vitro*, and fitted to the Partridge & De Grip template. An excellent fit was seen, and in digitonin extract the spectrum was red shifted by approximately 1 nm relative to the spectrum in situ. λ_{max} variation within one species was less than 1 nm, except for in *Rana temporaria* where intraspecific variations were larger (502.3–504.4 nm).

Second, to see if A1 visual pigments with extreme λ_{max} agree with the Mansfield transformation (absorbance plotted against normalized frequency ν/ν_{max}), amphibian green rods, transmuted gecko rods, and cones in a fish *Danio aequippinnatus* were recorded, and fitted to the Partridge template. It was concluded that the long-wave limb of all visual pigments peaking below 500 nm deviates consistently from the template, with deviations being larger, the smaller the λ_{max}. In order to correct for this insufficiency, the Mansfield transformed expression for the a-band, eq. (5) repeated here for clarity,

$$S(x) = 1/\{\exp[a(A-x)] + \exp[b(B-x)] + [c(C-x)] + D\} \quad (5)$$

was modified. Lambs original parameter values (A = 0.880, B = 0.924, C = 1.104 and D = 0.655, a = 70, b = 28.5 and c = –14.1) were adjusted to A = 0.8775, B = =0.9232, C = 1.1, D = 0.639478, a = 71, b = 27.5, c = –14.3 to give an even better fit to the bovine rhodopsin data. Parameters A and a define the slope and the Y-intercept of the line describing the fall in log sensitivity with λ_{max}/λ in the deep red part of the spectrum. They should be constant according to the Mansfield transform but since spectral invariance does not hold, a was set to 69.11 while A was redefined as a function of λ_{max}:

$$A = 0.877 + 0.0463 \quad \exp[-(\lambda_{max}-300)^2/11940] \quad (7)$$

In this modified version, A is virtually con-

stant for $\lambda_{max} > 500$ nm, and starts growing progressively below that, increasing the long-wave slope.

Third, A2 visual pigments (of carp *Cyprinus carpio*, sturgeons *Acipenser güldenstädti* and *A. baeri x A. medirostris*, and *A. stellatus*) were recorded and fitted with the Mansfield transform of a template based on carp porphyropsin (Bridges, 1967). The template worked well for visual pigments with λ_{max} inside the range 440–540 nm, however, highly red sensitive visual pigments (i.e. pigment 564 of *A. stellatus* and red cone pigment of carp) produced narrower curves than the expected. However, the following modifications to eq. (5) gave a good fit to the A2 visual pigment spectra: B = 0.9075, b = 20.91, C = 1.109, c = –10.4, D = 0.536 and

$$a = 62.9 + 1.84 \cdot \exp[(\lambda_{max}-623)/54] \quad (8)$$

$$A = 0.8723 + 0.0267 \cdot \exp[(\lambda_{max}-663)/40.6] \quad (9)$$

After subtracting eq. (5) with the corresponding parameter modifications from the complete experimental spectra of the A1 and A2 visual pigment, the β-band remained, which was fitted with a Gaussian. The β-band maximum and bandwidth were found to be linearly related to λ_{max} in A1 pigments. In A2 pigments the β-band maximum only, was linearly related to λ_{max}.

The modification affects the determination of λ_{max} from the long-wave slope (MacNichol, 1986). Instead of $\lambda_{max} = \lambda_{0.5}/1.0925$, the expressions for A1 and A2 visual pigments are given by

$$\lambda_{max} = 42.14 + 0.7683 \cdot \lambda_{0.5} + 0.000128 \cdot \lambda_{0.5}^{2} \quad (10)$$

and

$$\lambda_{max} = 37.78 + 0.7562 \ \lambda_{0.5} + 0.000135 \cdot \lambda_{0.5}^{2} \quad (11)$$

where $\lambda_{0.5}$ is the wavelength at which sensitivity is half maximal, $\lambda_{0.5} > \lambda_{max}$.

5.2. Spectral sensitivity and thermal activation rate in rods of two toad species (II)

The rods of the two toad species *Bufo bufo* and *Bufo marinus* were spectrally characterized with greater precision than earlier. My MSP -recordings suggest the λ_{max} of *B. marinus* rods is displaced by 1.5 nm towards longer wavelengths in relation to *B. bufo* rods. The best fitting template gave $\lambda_{max} = 501.7$ for *B. bufo* and $\lambda_{max} = 503.3$ for *B. marinus*.

Thermal activation in terms of quantal deviations in the membrane current of single rods in total darkness in the two species was similar, giving a rate of approximately 0.02 sec^{-1} for both species.

These estimates of dark noise in the two toad species deviate clearly from that estimated in spectrally similar rhodopsin rods in the frog *Rana catesbeiana* (<0.005 sec^{-1}, except 0.006 sec^{-1} for one cell, Donner *et al.*, 1990, $\lambda_{max} = 501.6$ nm).

5.3. Comparisons of the primary structures of six anuran rhodopsins (III)

The deduced nucleotide sequences of one new frog rhodopsin (*Rana temporaria*) and two toad rhodopsins (*Bufo bufo* and *B. marinus*) showed a high degree of identity with rhodopsin sequences in the GenBank database. The new rhodopsins were compared to previously sequenced anuran rhodopsins (*Rana catesbeiana* (Kayada *et al*., 1995), *Rana pipiens* (Pittler *et al*., 1993) and *Xenopus laevis* (Batni *et al*., 1996). At the amino acid level identity scores were of the range 96% within the genera, and as high as 90% between the genera, in comparison to 97% and 86% respectively at the level of nucleotides. Thus, the rate of silent to non-silent mutations in the nucleotide sequences is higher between different genera in comparison to species within one genus.

The number of amino acids that constitute the rhodopsin molecule differs from one class of animals to another. Mammalian rho-

dopsins are typically constituted by 348 amino acids (*Bos taurus* P02699, *Canis familiaris* P32308, *Homo sapiens* P08100), birds by 351 (*Gallus gallus* P22328), reptiles by 352 (*Alligator mississippiensis* U23802), and fish by >350 amino acids (354 in *Carassius auratus* P32309, 352 in sand goby *Pomatoschistus minutus* P35403). All toad and frog rhodopsins are 354 amino acids in length. The additional amino acids are located at the carboxy terminal of the molecule.

In the three new frog and toad rhodopsin sequences functionally important residues such as Glu^{113}, Lys^{296}, Cys^{110}, Cys^{187}, His^{65}, His^{152}, His^{211}, Phe^{115}, Ala^{117}, Glu^{122}, Trp^{126}, Ser^{127}, and Trp^{265} are all conserved. At locations suggested to influence absorbance characteristics of the molecule (listed in Table 1, section 2.5.2.), there are no non-conserved substitutions across the species, except at positions 83 and 299. Asparagine instead of aspartate at position 83 reportedly shifts spectral sensitivity towards blue by 8.5 nm (Nathans, 1990). In all anuran sequences position 83 is occupied by an asparagine.

There is a total of 52 amino acid substitutions across the six anuran species. Out of them, sixteen are non-conserved, and six include gain or loss of a hydroxyl group (listed in Table II, p. 301 in paper III). Ten of these 16 + 6 substitutions are located in the transmembrane helices. The following generic differences and similarities were observed: in comparison with the two *Bufo* species and *X. laevis*, the three investigated species of the genus *Rana* all have Lys instead of Gln at position 36, Phe instead of Val/Ile at position 112, and Thr instead of Phe at position 220. *Rana* species and *X. laevis* all have His100Asn, Gly149Ser, Tyr270Ser, Tyr274Phe, and Ala299Ser. Phylogenetic analyses of all sequences place *X. laevis* on a branch separate from that of the *Bufo* and *Rana* species.

5.4. E_a estimates in different visual pigments (IV)

Relative sensitivity in the long wavelength part of the spectrum at two temperatures was monitored in the main rod population (≅ 502 nm) and in red-sensitive cones (562 nm) in the frog *Rana temporaria*. Estimates of E_a from the sensitivity differences at the two respective temperatures, "cold" (5.4 °C) and "warm" (25.0 °C), gave 45.7 ± 0.4 kcal mol^{-1} for rods and 45.5 ± 0.4 kcal mol^{-1} for cones. The difference between the two estimates is significantly smaller ($P < 0.0005$) than the 11% expected if E_a were proportional to $1/\lambda_{max}$. Next, rods of the toad *Bufo bufo* (λ_{max} ≅ 502 nm) gave E_a estimates of 49.2 ± 0.6 kcal mol^{-1}, which is significantly higher ($P < 0.01$) than that estimated in frog rods.

To test the effect of changing the chromophore in one and the same opsin, *Rana temporaria* tadpole red cones (λ_{max} 629 nm) with approximately 40% A2 were investigated. E_a estimates gave 40.4 ± 1.6 kcal mol^{-1}, which is significantly ($P < 0.05$) lower than in frog adult cones with only A1. In addition, red cones (λ_{max} 634 nm) and rods (λ_{max} 528 nm) of *Xenopus laevis* with A2 were investigated, suggesting $E_a \lesssim 39$ kcal mol^{-1} and $E_a = 41.2 \pm 1.5$ kcal mol^{-1}, respectively.

Put together, these results show that $1/\lambda_{max}$ and E_a are not linearly related, and indeed they indicate no clear correlation at all between the two. However, they do suggest a generic difference in E_a between A1- and A2-based visual pigments. The results are summarized in Table III together with dark noise, E_a and λ_{max} data from the literature.

6. Discussion

6.1. A universal template for A1 and A2-based visual pigments (I)

Why is so much effort expended on looking for the most accurate template possible for visual pigment absorbance, and why is universality of the template desirable?

The true peak in a spectral sensitivity function, or absorbance curve, is difficult or impossible to determine, unless one takes into account the shape of the underlying function. Therefore, determining λ_{max} always goes via fitting a template to the experimental values, and a subsequent estimation of where the peak of the template is positioned. Therefore, different values of λ_{max} for the same visual pigments, reported by different authors, may be due to different ways of estimating λ_{max}, rather than due to visual pigment polymorphisms. A universal template would facilitate comparison of visual pigment absorbance characteristics across cell types, species and experimenters, also from noisy or limited data.

The universality of existing templates has been repeatedly challenged (Palacios *et al*., 1998; Kraft, 1988). The present investigation, which includes 39 different rod and cone types in amphibians, reptiles and fish, lends support to the idea of universality, with some modifications of the existing templates. For A1 visual pigments the template based on bovine rhodopsin extract by Partridge and De Grip (1991), and mathematically described by Lamb (1995) supplemented by modifications (equation (7)), predict absorbance rather well for visual pigments covering a wide range of λ_{max}. The modification of Lamb's expression corrects for the regular deviation of the long-wave limb of the template seen in visual pigments with λ_{max}<440 nm. For A2 visual pigments, similar modifications of the Lamb template (equations (8) and (9)), based on difference spectra obtained from por-

Table III. Spectral and thermal properties of some visual pigments

	cell type & **chromophore**	λ_{max} (nm)	dark noise (rod^{-1} s^{-1})	activation rate constant. (s^{-1})	E_a, light reaction (kcal mol^{-1})	E_a, thermal reaction (kcal mol^{-1})
Bb	rod A1	501.7	0.02[1]	10^{-11}	49.2 0.6	
Bm	rod A1	503.3	0.021[2]	10^{-11}		22
	green rod A1	432	0.065[3]	4.6 x 10^{-11}		20.1
Rc	rod A1	501.6	0.006[4]	0.15 x 10^{-11}		
Rc	rod A2	523	0.057[4]	1.2 x 10^{-11}		
Rt	rod A1	503.4			45.7 0.4	
Rt	cone A1	562			45.5 0.4	
Rt, juv	cone A2	629			40.4 1.6	
Xl	rod A2	523	0.015[5]	2.1 x 10^{-11}	41.2 1.5	
Hh	rod A2	538	0.053[6]	7 x 10^{-11}		
Ab	rod A2	549	0.09[6]	1.07 x 10^{-10}		

[1] Paper II
[2] Baylor et al., 1980, at 20°C
[3] Matthews, 1984, at 20°C
[4] Donner et al., 1990
[5] Donner et al., 1997
[6] Firsov & Govardovskii, 1990, at 19°C

Species key: Bb: *Bufo bufo*; Bm: *Bufo marinus*; Rc: *Rana catesbeiana*; Rt: *Rana temporaria*; Xl: *Xenopus laevis*; Hh: *Huso huso* x *Acipenser nudiventris*; Ab: *Acipenser baeri*; Ag: *Acipenser güldensädti*; Ab x: *A. baeri* x *A. mediostris*; Cc: *Cyprinus carpio*; Ca: *Carassius auratus*.

phyropsin extracts (Bridges, 1967), correct for deviations in the long-wave limb in the template seen in visual pigments with λ_{max}>550 nm. Deviations from the modified templates may be due to mixtures of different chromophores or more than one opsin expressed in the same cell.

Two reservations to the universality of the templates are the unknown influences of pH and anions in physiological conditions (which have been observed in experimental conditions in long-wave sensitive cones (Fager & Fager, 1979; Crescitelli 1980, 1981; Novitskii *et al*., 1989)), and the (λ_{max} -independent) behaviour in the long wave slope observed in both cones and rods as the availability of thermal energy is increased (paper IV).

According to the present investigation, absorbance spectra *in situ* and *in vitro* are fully comparable, with a regular hypsochromic shift of the spectrum *in vitro* by approximately 1 nm. The modified versions of A1 and A2 visual pigment spectral templates (equations (7), (8), (9)) fit available (electrophysiological) spectral sensitivity data well. An exception is the short-wave region (<440 nm) where absorbance tends to be higher than spectral sensitivity.

As high quality MSP recordings in cones are difficult to obtain (due to small cell size), and due to the fact that cone visual pigments are more sensitive than rods to the ionic milieu, detergents, etc., templates generated from rod absorbance *in vitro* and *in situ* were fitted to cone spectra, with the assumption that they are comparable.

6.2. Thermal activation rates compared in two toad species (II)

The idea that thermal reactions intrinsic to the eye, i.e. thermal activation of visual pigments in the photoreceptors, may constitute an inexorable limit to retinal threshold (Fechner,1860; Autrum, 1943; Barlow ,1956 & 1957), has gained wide acceptance along with support from experimental work (Ashmore & Falk, 1977; Ashmore & Falk 1982; Baylor *et al*., 1980; Copenhagen *et al*., 1987; Aho *et al*., 1988 & 1993a). However, the surprisingly high thermal stability of rhodopsin in rods of the frog *Rana catesbeiana* (Donner *et al*.,1990) suggested that pigment activation rates could be so low that their biological importance is questionable. This finding raised further questions about the interspecies variability of rhodopsin stability.

Visual thresholds of the European toad *Bufo bufo* have been thoroughly determined in behavioural experiments and recordings from retinal ganglion cells (Aho *et al*., 1988, 1993a), but these studies lacked direct information on thermal activation rates in rods of the same species. The purpose of paper II was to test the assumption that the rods of *Bufo bufo* have similar noise properties as the spectrally similar rods of the cane toad *Bufo marinus*. For the latter species, Baylor *et al*. (1980) had reported a thermal activation rate of 0.028 rod^{-1} sec^{-1} at 20 ºC and 0.015 at 15 ºC, which would be consistent with the *Bufo bufo* behavioural threshold at the same temperature (0.01-0.02 photoisomerizations rod^{-1} sec^{-1}). We recorded about 0.020 dark events per rod and per second, at 20º C, in *Bufo bufo*. As the rods are of approximately the same size, the rates do not differ significantly in the two species. This supports the notion that thermal dark events in rods limit visual threshold in *Bufo bufo*.

6.3. The primary structure of rhodopsin (III)

Gene technology of today and the natural variation in λ_{max} between animal species offer an exceptional opportunity to compare the genes coding for visual pigments, pin-point amino acid differences between them, and test their spectral influences. According to several investigations (Nathans, 1990; Nakayama & Khorana, 1990; Merbs & Nathans, 1993; Asenjo *et al*., 1994) either non-conserved amino acid substitutions, or gain or loss of a hydroxyl group in the transmembrane portions of the molecule, are most likely to influence the absorbance char-

acteristics of visual pigment molecules.

Since the investigated *Rana* and *Bufo* rhodopsins have very similar λ_{max}, the set of non-conserved substitutions found across their amino acid sequences have no substantial influences on spectral sensitivity. In the frog and toad sequences, there are amino acid changes at two positions that have been assigned spectral tuning properties. In both frogs and toads there is an asparagine instead of aspartate at position 83, which according to the literature, produces 8 nm blue shifts in bovine rhodopsin (Nathans, 1990). The set of amino acids in the anuran sequences must counteract this blue shift, since they are slightly red-shifted respective to bovine rhodopsin (λ_{max} 498 nm (Nathans, 1990)). The toad (*Bufo*) rhodopsins have a serine instead of alanine at position 299, which produces a small red shift (Fasick *et al.*,1998), which probably in part counteracts the blue shift by Asn83 in toad rhodopsins. In frogs (*Rana*) that are equally red sensitive as the toads, the blue shift by Asn83 must be counteracted by other amino acids. Consequently, frog and toad rhodopsins may achieve similar absorbance spectra with different sets of amino acids. This may bring about the observed differences in dark activity between the species (Donner *et al.*, 1990, paper I).

Even though spectral shifts may be compensated, it might be that one set of amino acids gives higher thermal stability than another. Spectral shifts towards shorter wavelengths coupled to *increased* dark activity of the pigment have been reported following two types of experimental modifications: upon introduction of large amino acid side chains at position 121 in rhodopsins (Han *et al*. 1996) and upon regeneration of salamander rod pigment with the chromophore 4-hydroxy retinal (Corson et al., 1990). In salamander, interactions between the chromophoric ring and opsin produce spectral shifts in red and blue cones, but not in rods (Makino *et al*., 1999). One could speculate that perturbation of the chromophoric ring could still influence the thermal stability of the rod visual pigment.

The rhodopsin sequences that are compared were obtained from closely related amphibian species, with rods having similar absorbance characteristics, but reportedly different rates of dark events. Out of a total of 52 substitutions across the sequenced rhodopsins of six amphibian species (*Bufo bufo, Bufo marinus, Rana catesbeiana, Rana pipiens, Rana temporaria, Xenopus laevis*) there are 16 non-conserved substitutions and 6 that include gain or loss of a hydroxyl group (Table II, p. 301, paper III). As the thermally stable rhodopsin of the bullfrog, *Rana catesbeiana*, contains no unique, important mutations compared with the other two species of the genus *Rana*, the survey of mutations that follows, starts from the assumption that all *Rana* species may have thermally stable rhodopsins.

The three investigated species of the genus *Rana* have in common phenylalanine instead of valine at position 112, serine or threonine instead of phenylalanine at position 220, tyrosine instead of serine at position 270, and alanine instead of serine at position 299 in the transmembrane regions. These residues are located in helices III, V, VI and VII, respectively. According to the model of rhodopsin proposed by Pogozheva *et al*. (1997), transmembrane helices III and VI would form a significant portion of the pocket, parts of them being in close proximity to the chromophoric ring.

In a comparison of the rhodopsins from a selection of mammals, birds, reptiles and fish, it appears as though phenylalanine at position 112, serine or threonine at 220 and tyrosine at 270 (in the genus *Rana*), would not be found in warm-blooded animals. Moreover, serine at position 299 is found only in warm-blooded animals, except for the two toad species (Table IV).

In helix III, valine at position 112 in the toad rhodopsins is substituted by the bulky phenylalanine in the frog rhodopsins. Generally, this is a variable site in vertebrate rhodopsins, however, it could have some functional consequences due to its immediate proximity to glutamate[113], which serves as counterion to the Schiff-base linkage between retinal and opsin.

Table IV. Comparing amino acid substitutions at positions 112, 220, 270, 274 and 299 in rhodopsins of a selection of animal species.

Position: 112, helix 3.
F: frogs (*Rana*), alligator
I: anolis, chicken, lamprey, Xenopus, ambystoma
L: bovine, carp, hamster, human, mouse, sheep, 11 Baikal cottoids V: toads (*Bufo*), dog, sand goby, rabbit
A: macaca
P: gold fish

Position: 220, helix V.
S or T: frogs (*Rana*), *Cottocomephorus inermis*
F: toads (*Bufo*), and all others

Position: 270, helix 6.
S: toads (*Bufo*), alligator, anolis, dog, carp, chicken, goldfish, sand goby, human, lamprey, macaca, mouse, rabbit, Ambystoma, 11 Baikal cottoids
G: bovine, hamster, sheep
Y: frogs (*Rana*), *Xenopus*

Position: 274, lumenal loop 3.
Y: *Rana*, all others
W: 10 Baikal cottoids
F: toads (*Bufo*)

Position: 299, helix 7.
A: frogs (*Rana*), alligator, anolis, bovine, carp, chicken, hamster, goldfish, sand goby, human, lamprey, *Xenopus*, *Ambystoma tigrinus*, 12 Baikal cottoids
S: toads (*Bufo*), dog, macaca, mouse, rabbit, sheep

The selected rhodopsin sequences were obtained from Hargrave & McDowell (1992), Smith *et al*. (1995), Archer *et al*. (1992) and Hunt *et al*. (1996).

In helix V there is a net gain of a hydroxyl group at position 220 in frogs: serine or threonine in *Rana* rhodopsins is substituted by phenylalanine in *Bufo* rhodopsins. The hydrophilic and highly reactive side chains of serine and threonine may influence the reactivity with water molecules, which again may have some consequences for thermal stability in the rhodopsin molecule. Phenylalanine is found at this position in all animals screened by this study (table IV), except for in the three frog species of the genus *Rana*, and in one Baikal cottoid species (*Cottocomephorus inermis*) (Hunt *et al*., 1996). In human rhodopsin, there is a cysteine instead of phenylalanine at position 220 in a number of families with autosomal dominant retinitis pigmentosa (Bunge *et al*., 1993). The heritable retinal disorder retinitis pigmentosa is characterised by slow progressive degeneration of the peripheral retina.

Position 299 in helix VII is located towards the interior of the retinal binding pocket (Baldwin, 1993; Pogozheva *et al.*, 1997) and may therefore be in direct interaction with the chromophore. It is presumably situated close to the Schiff-Base linkage between the opsin and the chromophore. The substitution of alanine by glutamate at this position brings about a 15 nm red shift (Weitz and Nathans, 1993), cysteine produces a blue shift (Lin *et al*., 1998), and serine instead of alanine brings about a small red shift (Fasick *et al.*, 1998). In nature there is either an alanine or a serine at this position in rhodopsins (Table IV), which involves gain

or loss of a hydroxyl group.

The presence or absence of a hydroxyl group at position 299 could produce influences on the pkA-value of the Schiff-base linkage between the chromophore and the opsin, and thereby, perhaps, thermal stability of the visual pigment. Interestingly, in animal species with rhodopsins that may bind both A1 and A2, or A2 only (*Rana pipiens* (SP:P31355), *Rana catesbeiana*, *Rana temporaria*, *Xenopus laevis* (SP:29403), *Ambystoma tigrinum*, *Carassius carassius* (Comp. Biochem. Physiol 109B:81–88 (1994)), *Pomatoschistus minutus* (SP: P35403), *Carassius auratus* (SP:32309) *and Petromyzon marinus* (SP:P22671)), there is exclusively an alanine, and hence, no hydroxyl group at position 299. A2-based visual pigments are red-shifted in a regular manner compared to the corresponding A1 pigments.

Thermal dark events are more frequent (Donner *et al*., 1990), and energy barriers of activation are significantly lower in porphyropsin rods in comparison to rhodopsin rods (paper IV). Hence, the A2 chromophore might put particular demands on the stabilizing properties of the opsin.

In order to test the possible effects on thermal stability of the amino acid substitutions found in the frog rhodopsin sequences, mutagenesis could be performed, involving at least residues 299 and 220, and possibly 112 and 270, followed by expression in transgenic animals and thermal studies. At present, transgenic *Xenopus laevis* have been successfully produced, however, rhodopsin knock-out frogs are still on the waiting list (B. Knox, personal communication).

6.4. Spectral sensitivity and activation energy estimates of a number of visual pigments (IV)

The thermal contribution to visual excitation has two important functional consequences. One is that the spectrum of light available for vision is extended towards longer wavelengths. According to the present results, this can be achieved either by lowering the energy barrier of activation (as by a substitution of the chromophore A1 with A2), or by recruiting thermal energy for visual excitation with greater efficiency, as in frog red cones compared with rods. Lowering of the energy barrier in visual pigments with A2 chromophore is expected from the extra double bond of 3-dehydroretinal (Williams & Milby, 1968). The molecular mechanisms of a possible high capacity to recruit thermal energy towards chromophore isomerization remain to be explained.

The second consequence of thermal contribution to excitation in visual pigments is susceptibility to activation by thermal energy alone, which would generate dark noise in photoreceptors. The relative red-sensitivity of A2-based visual pigments really seems to correlate both with lower activation energy and lower thermal stability than in their A1 counterparts (Bridges, 1967; Williams & Milby, 1968; Donner *et al*., 1990). According to some investigations, the "efficient thermal energy recruiters", cones, appear to have high rates of thermal activation (Lamb & Simon, 1977; Schnapf *et al*., 1990, Donner, 1992).

The results of this paper show that there is no necessary relation between λ_{max} and E_a in visual pigments (Fig. 3). Barlow (1957) originally proposed a direct relationship between the barrier of activation and peak absorbance of a visual pigment ($E_a = hc/\lambda_a$): sensitivity to low energy photons would imply a lower energy barrier of activation (and presumably more dark noise). His arguments were convincing, and it is indeed surprising that visual pigments do not obey these simple principles. A number of recent experimental studies have been taken to support the generally accepted idea that spectral tuning through structural changes in the opsin involves a change in the transition energy of the chromophore, lowering along with red shifts of spectral sensitivity (Kochendoerfer *et al*., 1997 & 1999; Kosower, 1988). On the other hand, Han et al. (1996) have shown that substituting Gly121 by a number of amino acids (G121A, Ser, Thr, Val, Ile, Leu, and Trp) both blue shifts the visual pigment, and increases its activity

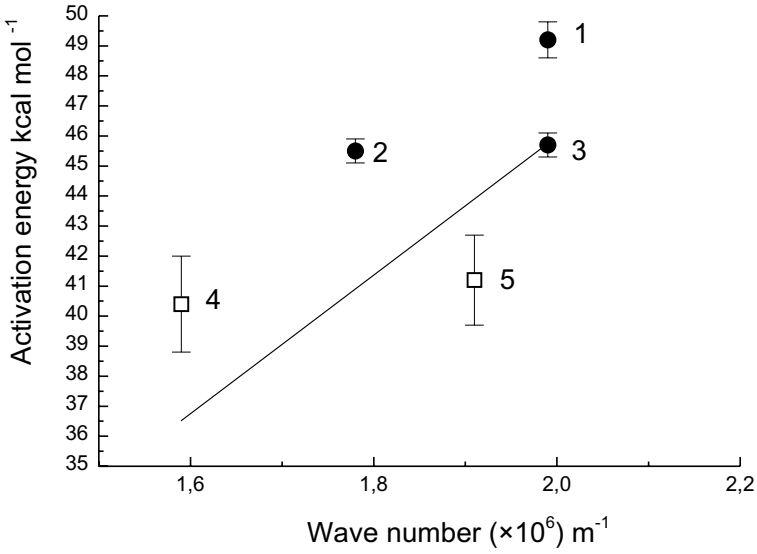

Figure 3. E_a estimates in visual pigments with different λ_{max} (in wavenumbers). 1: red rods in the toad *B. bufo* (A1); 2: red cones in the frog *R. temporaria* (A1) 3: red rods in the frog *R. temporaria* (A1); 4: red cones in the frog *R. temporaria* tadpole (A2); 5: red rods in the frog *X. laevis* (A2). B) The line illustrates where E_a values would fall relative to that measured in frog red rods, if $E_a \propto 1/\lambda_{max}$.

(transducin activation) in the dark, which would suggest a lowering of the energy barrier of activation.

Factors that may influence spectral properties and activation energy include the structure of the chromophore, various amino acids that interact directly with the chromophore, and ions, such as Cl^-, which is important in the spectral tuning of cone pigments (e.g. Kleinschmidt & Hárosi, 1992). Furthermore water molecules in the retinal binding pocket may contribute.

It should be noted that the E_a estimates for A1 pigments are more than two times higher than those obtained from temperature-dependent thermal activation rates in toad rhodopsin rods (Baylor *et al*., 1980). This supports the notion that activation by light and activation by thermal energy alone proceeds along different molecular routes (Barlow *et al*., 1993).

To further investigate the relation between E_a, λ_{max}, and dark noise, blue sensitive visual pigments such as those of blue cones and "green" rods should be included in future studies. Possible generic differences between frogs and toads could be analysed, and spectral sensitivities of poikilotherms could be compared to that of mammals at different temperatures.

Summarizing, I propose that in analyses of the molecular mechanisms of spectral tuning, two different physical possibilities should always be considered: either a changed energy barrier for activation, or a changed capacity for directing vibrational energy towards chromophore isomerization.

In Fig. 4 frog rod spectra are superimposed on monkey (Baylor et al., 1984) and human (Kraft et al., 1993) rod spectral sensitivity obtained from suction pipette recordings. In the deep red part of the spectrum, frog rod sensitivity (both as recorded at 5°C and 25°C) declines faster than human and monkey rod sensitivities (recorded at 37 °C). This suggests that the visual pigments of mammals may be similarly susceptible to the actions of thermal energy. However, the comparison also suggests that mammalian long-wavelength sensitivity might decline more steeply than that of frogs if measured at the same temperature. This might indicate some structural protection against heat in mammalian pigments.

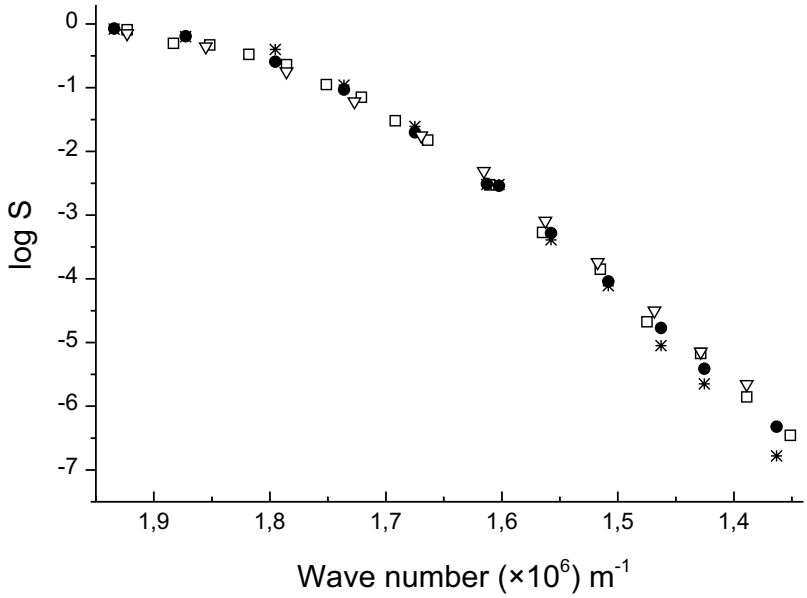

Figure 4. Warm (●) and cold (✳) spectra of frog rods compared to monkey (▽ Baylor *et al.*, 1984) and human rods (□ Kraft *et al.*, 1993).

7. Conclusions

The contribution of thermal energy to visual excitation brings about two functional consequences for vision. One is that sensitivity is extended in the longwave part of the spectrum, where photon energy is lower than the energy barrier of excitation in visual pigments. Another is that thermal energy may produce visual pigment excitations in the absence of light. Thermal reactions decrease signal-to-noise ratios in photoreceptors, and may thereby limit retinal threshold.

According to a long held notion, the energy barrier of excitation would be inversely proportional to the wavelength of maximum absorbance of visual pigments. This would imply a higher degree of noisiness in photoreceptors sensitive to very long wavelengths. Thus, generating highly red-sensitive visual pigments, even in long wave dominated light environments, would be undesirable due to the need to maintain reasonable retinal signal-to-noise ratios. Red-sensitivity "avoidance" can be observed in nature in the rhodopsins of terrestrial vertebrates in general, and in fresh water fish and amphibians in particular.

These investigations challenge the idea of a necessary and unconditional functional coupling between spectral sensitivity and thermal properties, i.e. the energy barrier of activation and dark noise in visual pigments. Instead, these functional properties may be independently tuned by mutations in the opsin molecule. However, in spectral tuning accomplished by switching chromophores from A1 to A2, the principle of interdependent physical properties appears tenable.

A first step towards tying function to protein primary sequence is to compare the sequences of phylogenetically close rhodopsins housed by photoreceptors that have been functionally surveyed. We have pin-pointed at least four candidate amino acids for future site-directed mutagenesis experiments. Ideally, spectral and thermal studies might be conducted on photoreceptors of transgenic animals in which the wild type pigments have been "replaced" by the mutant pigment.

The spectral properties of a number of visual pigments are analyzed. A template is proposed which applies to pigments spanning a wider range of spectral maxima and describes absorbance with improved accuracy.

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