Early Photolysis Intermediates of Gecko and Bovine Artificial Visual Pigments†

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ABSTRACT: Nanosecond laser photolysis measurements were conducted on digitonin extracts of artificial pigments prepared from the cone-type visual pigment, P521, of the Tokay gecko (*Gekko gekko*) retina. Artificial pigments were prepared by regeneration of bleached gecko photoreceptor membranes with 9-*cis*-retinal, 9-*cis*-14-methylretinal, or 9-*cis*-α-retinal. Absorbance difference spectra were recorded at a sequence of time delays from 30 ns to 60 μs following excitation with a pulse of 477-nm actinic light. Global analysis showed the kinetic data for all three artificial gecko pigments to be best fit by two-exponential processes. These two-exponential decays correspond to similar decays observed after photolysis of P521 itself, with the first process being decay of the equilibrated P521 Batho ⇔ P521 BSI mixture to P521 Lumi and the second process being the decay of P521 Lumi to P521 Meta I. In spite of its large blue shift relative to P521, iso-P521 displays a normal chloride depletion induced blue shift. Iso-P521's early intermediates up to Lumi were also blue-shifted, with the P521 Batho ⇔ P521 BSI equilibrated mixture being 15 nm blue-shifted and P521 Lumi being 8 nm blue-shifted relative to the intermediates formed after P521 photolysis. The blue shift associated with the iso-pigment is reduced or disappears entirely by P521 Meta I. Similar blue shifts were observed for the early intermediates observed after photolysis of bovine isorhodopsin, with the Lumi intermediate blue-shifted 5 nm compared to the Lumi intermediate formed after photolysis of bovine rhodopsin. These shifts indicate that a difference exists between the binding sites of 9- and 11-*cis* pigments which persists for microseconds at 20 °C.

Our knowledge of visual pigment photochemistry and function is largely based on studies of the rhodopsins from rod cells which function at low light levels. In contrast, at high light levels cone visual pigments generate the excitation signal, not rhodopsin. Higher ambient light levels intrinsically carry more information that cone cells and their pigments are designed specifically to exploit. Cone cells are usually smaller than rods, providing higher spatial resolution and faster time response. Similarly, their pigments are tuned to have different spectral sensitivities in specific cone cells to extract color information; cone pigments could also have to deal with the issue of secondary photolysis which can arise at higher illumination levels. Both of these pigment features point to significant differences between rod and cone pigments which make it unlikely that an adequate understanding of the latter can be based solely on experiments with the former. For example, the late photointermediates (beginning with Meta I)[1] of most rhodopsins absorb in the visible region, making those pigments unsuitable for vision at high light levels because their accumulation would lead to secondary photoproducts. Indeed, what little is known of the late photointermediates of cone pigments indicates differences in exactly this aspect. Similarly, regulation of color sensitivity in cone pigments is central to their function, but the mechanism for this is also poorly understood. Studies of point mutations in bovine rhodopsin show many ways that its absorbance spectrum can be changed. However, most are associated with functional problems, as for example the G90D mutation, leaving open the question of how nature controls absorbance of pigments in functionally benign ways (*1*).

Studies of cone pigments have been comparatively rare because cone pigments usually occur in mixtures, and separation of the pigments is difficult due to the fact that they are chemically less stable than rhodopsins. It is thus extremely fortunate that over 90% of the visual pigment in the Tokay gecko retina consists of a single cone-type pigment, P521 (*2*, *3*). P521 displays several features which are important for the understanding of wavelength regulation and function of cone pigments. One cone pigment property shown by P521 is a chloride-induced red shift of approximately 20 nm in its long-wavelength absorbance band. Another property which differs between cone pigments and rod pigments is the extremely large blue shift of the 9-*cis* (iso)pigments relative to those formed from the native 11-*cis* chromophore. In P521 this shift is 33 nm compared to 13 nm for bovine rhodopsin (*4*). In the chicken red cone pigment, iodopsin, the shift from isoiodopsin to iodopsin has been reported to be even larger, on the order of 40 nm (*5*), but the shift is only this large near room temperature (*6*). On cooling to −195 °C the spectrum of the iso-pigment shifts until it is only 25 nm to the blue of iodopsin. Given these

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[1] Abbreviations: Batho, bathorhodopsin; Lumi, lumirhodopsin; Meta I, metarhodopsin I; PSB, protonated Schiff base; ROS, rod outer segment; TBS, TRIS-buffered saline solution; TRIS, tris(hydroxymethyl)aminomethane.

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interesting shifts in the cone pigment spectra induced by factors which have little or no effect on bovine rhodopsin, the question naturally arose of how these shifts correlate with those of the photointermediates of P521.

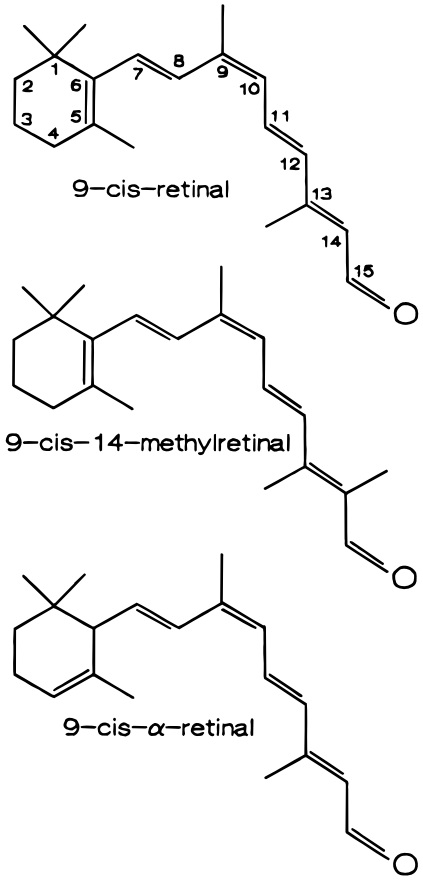
In previous work (*7*) we explored the chloride dependence of P521 photochemistry. For P521, decay of the Batho photoproduct is much faster than for bovine rhodopsin. At room temperature P521 Batho has relaxed into an equilibrated mixture with its decay product, P521 BSI, within 20 ns after photolysis. This equilibrated mixture was also seen after chicken iodopsin photolysis and was designated BL (*8*). On the 100-ns time scale the equilibrated mixture decays to P521 Lumi, and on the 5-μs time scale P521 Lumi decays to P521 Meta I.

$$\text{P521 Batho} \overset{K_{eq}}{\leftrightarrow} \text{P521 BSI} \rightarrow \text{P521 Lumi} \rightarrow \text{P521 Meta I ...}$$

Chloride concentration was found to affect the K_{eq} between P521 Batho and P521 BSI. The presence of chloride had been previously shown to cause thermal back-isomerization at low temperatures after photolysis of iodopsin (*6*). At room temperature the photolysis of P521 reduces the chloride binding constant at the BSI photointermediate. This implies that chloride release accompanies the normal progress of the early cone pigment photointermediates.

Here we report complementary investigations of the photointermediates of several artificial iso-pigments of P521 to gain further insight into the structural characteristics of early photointermediates of cone pigments. We regenerated gecko pigments using 9-*cis*-retinal, 9-*cis*-14-methylretinal, and 9-cis-α-retinal (with the double bond moved from the 5,6 position of the ring to the 4,5 position; Chart 1). One of these chromophores, 9-*cis*-14-methylretinal, had not been previously studied in its bovine pigment, and previous studies of bovine isorhodopsin had left uncertainties concerning the absorbance λ_{max} of its early photolysis intermediates, bathorhodopsin (Batho), BSI, and lumirhodopsin (Lumi). We thus report new studies of those bovine pigments, as well.

Chart 1: Artificial Visual Pigments[a]



[a] These pigments were prepared by regeneration with the aldehydes shown (all these chromophores produce iso-pigments since they are 9-*cis* isomers rather than the native 11-*cis*-isomer of retinal).

EXPERIMENTAL PROCEDURES

Sample Preparation

The synthesis of 9-*cis*-14-methylretinal was performed according to the method of Chan *et al.* (*9*). 9-cis-α-retinal was synthesized from α-ionone using methods described previously (*10*).

Purified gecko visual pigment membranes were prepared as follows. Thirty-six retinas were removed under dim red light (Kodak No. 1A safety lamp) from the eyes of Tokay geckos (*Gekko gekko*) which were dark adapted, immobilized by cooling to 4 °C, and then sacrificed. The retinas were homogenized in 43% sucrose in Buffer K (67 mM sodium phosphate buffer, pH 7.4, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF). Photoreceptor outer segment membranes were recovered from the homogenate by three flotations (39000*g* at 4 °C for 30 min) using 43% sucrose in Buffer K to maximize the recovery. The membranes were then subjected to two successive flotations using 38% sucrose in Buffer K, washed twice with Buffer K, and suspended in 10 mL of Buffer K.

To bleach gecko P521, photoreceptor membrane suspensions were illuminated in the presence of 100 mM NH_2OH at 4 °C for 3 min with yellow light from a 500 W tungsten lamp equipped with a Corning 3-73 cutoff filter. Regeneration of pigments proceeded typically as follows. The bleached membranes were washed once with Buffer K containing 1 mg/mL bovine serum albumin, twice with Buffer K, and then resuspended in 5 mL of Buffer K. The bleached pigments were then regenerated by addition of an approximately equimolar amount of chromophore and incubated at 4 °C for 1 h. The membranes were then pelleted, resuspended in Buffer K, and frozen for future use. Gecko preparations which had been regenerated with the appropriate retinal derivative were thawed and extracted with 2% digitonin dissolved in pH 7.0, 10 mM TRIS, 60 mM KCl, 40 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA buffer (TBS) as previously described (*7*). Typically 1.2 mL of extract was used to measure a series of time dependent difference spectra. Due to varying yields in regenerating the pigments the concentrations of the extracts differed, with typical values for the pigments being 1, 0.4, and 0.2 absorbance units/cm for the iso-pigment, 14-methyl-iso-pigment, and α-iso-pigments, at their λ_{max}values of 488, 490 and 455 nm, respectively.

Bovine rod outer segments (ROS) were regenerated with 9-*cis*-14-methylretinal using a procedure described previously (*11*). The regeneration gave a pigment absorbing at 492 nm with a yield of 86% based on the amount of opsin originally present. The regenerated ROS were stripped of extrinsic membrane proteins by washing with pH 7.0, 1 mM EDTA solution. After stripping, the pigment-containing membranes

were spun down and solubilized in 2% octyl-β-D-glucopyranoside detergent (octyl glucoside) in TBS. The resulting solution had a bleachable absorbance of 1.1 in a 1-cm path length cell at 492 nm. Bovine isorhodopsin was a gift of Steven Smith and May Han of the Department of Chemistry at Yale University. It was also solubilized in 2% octyl glucoside in TBS at a final concentration of 1 mg/mL.

Chloride Effect

To determine the dependence of the iso-P521 absorption λ_{max} on chloride concentration, a 100-μL aliquot of the 2% digitonin suspension was dialyzed against pH 7.0, 10 mM TRIS, 100 mM K_2SO_4 solution using a Slide-A-Lyzer dialysis cassette. The contents were diluted 1:10 into 2% digitonin in pH 7.0, 10 mM TRIS, 100 mM K_2SO_4, and the absorbance spectrum was recorded at room temperature in a Shimadzu 2101 spectrophotometer. Successive aliquots of 4M KCl solution were added and the spectrum was rescanned. The λ_{max} of each sample was determined relative to the spectrum recorded after bleaching in the presence of 5 mM NH_2OH.

Kinetic Spectroscopy

Time dependent difference spectra were measured as described previously (*7*) at a range of delays from 20 ns to 60 μs after photolysis with a 7-ns pulse of 477-nm light (fluence = 100 μJ/mm^2). Measurements on bovine isorhodopsin samples were conducted using an apparatus (24 μL sample per photolysis) which has a 1-cm probe beam path length (*12*). Measurements on all other samples studied here were conducted using a microscale apparatus (1 μL of sample per photolysis) with 2-mm probe beam path length (*13*). This apparatus was improved to allow temperature control, and all measurements reported here were performed at 20 °C. For either apparatus, the probe beam was polarized at 54.7° relative to the linearly polarized actinic laser.

The absorption difference spectra collected for each sample were fit globally using previously described methods (*14*). Fits to sums of one, two, and three exponentials were attempted. For a given fit, lifetimes and b-spectra (spectral changes associated with each lifetime) were obtained. Combined with an assumed mechanism, the b-spectra can be used to construct intermediate difference spectra which are the absorbance difference between the assumed mechanism's postulated intermediates and the "bleach", the spectrum of the pigment changed by photolysis. By adding the "bleach" spectrum to the intermediate difference spectra, absolute spectra of the intermediate species can be determined. To measure the "bleach" spectrum of the bovine pigments studied here, a previously described procedure was used (*15*). Because gecko P521 is unstable in the Ammonyx LO used for the bovine procedure, a modified procedure was used to remove absorbance of photoproducts from the wavelength region where the artificial gecko pigments' long-wavelength absorption bands occur. We have determined that at low concentrations of NH_2OH (<5 mM) the gecko pigments studied here are stable for the time required for the bleach measurement. For the gecko pigments, 3 mM NH_2OH was added to the special sample used to determine the "bleach"; in all other aspects the procedure was the same as previously used.

Table 1: Observed Lifetimes

species	Batho ⇔ BSI	[Batho ⇔ BSI] → Lumi	Lumi → Meta I
gecko P521[a]	*b*	110 ns	4.8 μs
gecko iso-P521	*b*	120 ns	7.9 μs
gecko 14-methyl-iso-P521	*b*	550 ns	23 μs
gecko α-iso-P521	*b*	60 ns	1 μs
bovine rhodopsin[c]	40 ± 7 ns	230 ± 20 ns	*b*
bovine isorhodopsin	30 ± 7 ns	210 ± 20 ns	*b*
bovine 14-methyl-isorhodopsin	60 ± 15 ns	410 ± 100 ns	*b*

[a] Lewis *et al*. (*7*). [b] Not observed [c] Data of Hug *et al*. (*14*) interpolated for 20 °C.

Wavelength Accuracy

The accuracy of the λ_{max} values reported for the photointermediates in Table 2 depend on a number of factors. In general, the highest accuracy is achieved for longer lived intermediates which have no significant back-reactions to the preceding intermediate. Where such equilibria are present, uncertainties in the equilibrium constant are the dominant contributor to the λ_{max} uncertainty. In favorable cases such as for Lumi in all pigments studied here and for Meta I in P521, the accuracy is limited by the precision with which the spectrograph can be reset to the same position (±2 nm). When better accuracy is required, as needed for confidence that a difference exists between the Lumi from bovine rhodopsin and isorhodopsin, this source of uncertainty was eliminated by conducting experiments on both pigments without resetting the spectrograph. Under those conditions the precision of measurements is ±1 nm and the uncertainty in the accuracy of the measured difference between λ_{max}-values of two intermediates is ±1.5 nm instead of the ±3 nm expected from the uncertainty in the accuracy of the independent measurements.

RESULTS

Gecko Photolysis Experiments

Data collected after photolysis of the three artificial gecko pigments are shown in Figure 1. For all three artificial pigments the data covering the range from 20 ns to 60 μs were best fit by two-exponential processes whose lifetimes (base e) are given in Table 1. As is the case for P521 (*7*), the decay of Batho into equilibrium with BSI is too fast to resolve with our 20-ns time resolution, and the first species observed in all three pigments is the equilibrated Batho ⇔ BSI mixture. The first observed lifetime corresponds to the decay of this mixture to Lumi, and the second lifetime corresponds to the decay of Lumi to Meta I. Figure 2 shows the absolute spectra of the three species formed for each of the artificial pigments. The light lines give the experimental spectra obtained from the global fit by adding back the bleach, and the heavy lines show the fit of simple Gaussians to the experimental spectra. Table 2 gives the λ_{max} values for these Gaussians. Since the spectrum of the first species formed after photolysis of the iso-gecko and iso-14-methyl-gecko pigments is very broad, indicating the presence of significant amounts of both Batho and BSI in the equilibrium mixture, the first species for these two pigments was fit to two Gaussians corresponding to Batho and BSI. The basis for the decomposition is discussed below.

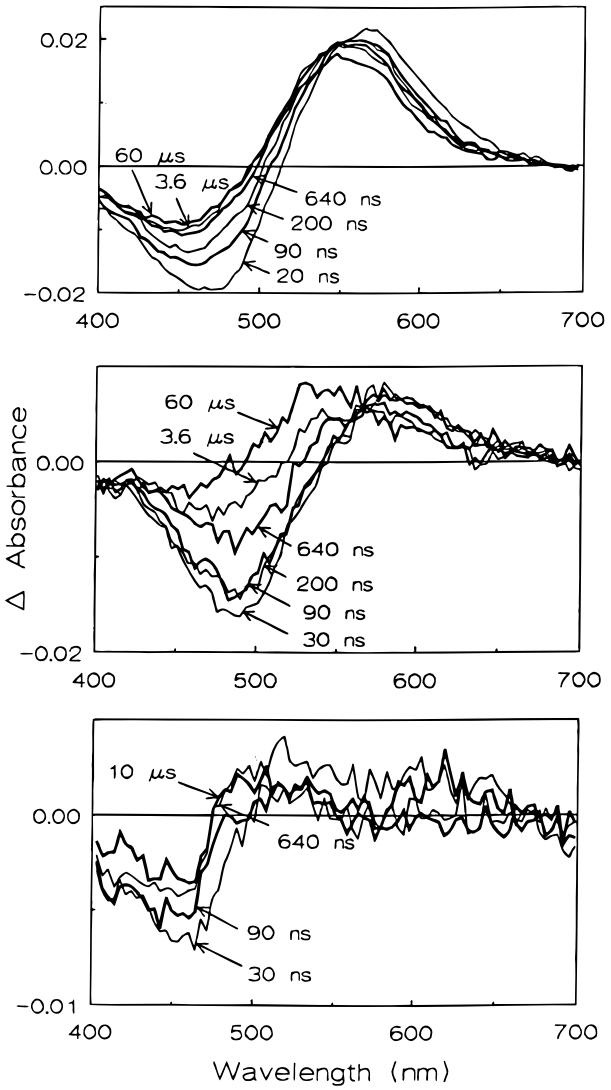


FIGURE 1: Absorbance difference spectra recorded at a series of time delays after 477-nm photolysis of gecko artificial visual pigment in digitonin extracts. (Top) Iso-P521. Averaged data from two experiments each using 1.2 mL of an extract of gecko retinas which had been regenerated with 9-*cis*-retinal and which had an absorbance of 0.2 at 488 nm in the 0.2-cm path length cell used here. (Middle) Iso-14-methyl-P521. Data from an extract of gecko retina which had been regenerated with 9-*cis*-14-methylretinal. The extract had an absorbance of 0.08 at 490 nm in the 0.2-cm path length cell used here. (Bottom) Iso-α-P521. Data from 1.2 mL of an extract of gecko retina which had been regenerated with 9-*cis*-α-retinal and which had an absorbance of 0.04 at 455 nm in the 0.2-cm path length cell used here. Data were collected at fewer time delays because the low sample concentration reduced the signal-to-noise ratio. These data are typical of those obtained from two different regenerations with this chromophore.

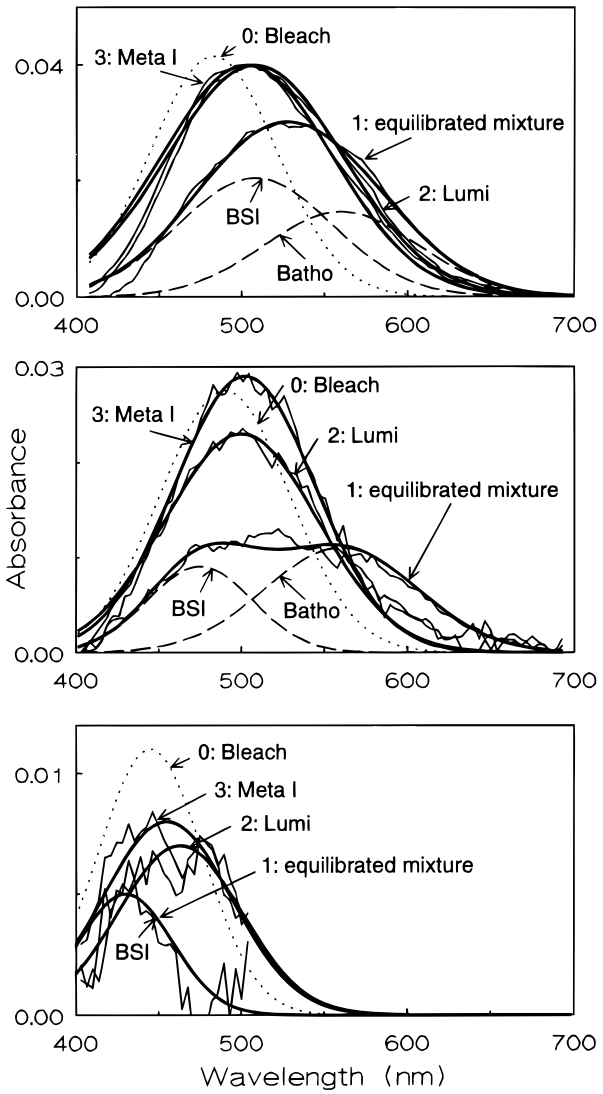


FIGURE 2: Spectra of intermediates observed after photolysis of gecko artificial visual pigments. (Top) Iso-P521. Data in Figure 1 (top) were best fit with a biexponential process which corresponds to a sequential decay involving three species whose specta are shown by the light lines. The spectra of the intermediates were fit to Gaussian functions shown by the heavy lines. The spectra of the equilibrated mixture was decomposed into the sum of two Gaussian functions shown by the heavy dashed lines labeled Batho and BSI. An equilibrium constant of 1.4 was used in the decomposition (assuming equal extinction coefficients for Batho and BSI). The λ_{max} for Gaussians is given in Table 2. (Middle) Iso-14-methyl-P521. Spectra for the intermediates computed as described above using an equilibrium constant of 0.5. (Bottom) Iso-α-P521. For this pigment noise prevented using all the data in the fit. Instead, only the wavelength region where the signal was largest was fit. The range fit is shown by the light lines. By analogy with the artificial bovine rhodopsin pigment made from 9-*cis*-α-retinal the first species observed is composed only of BSI.

Figure 2 also shows the Gaussians that were fit to the bleach experiments. While this spectrum is, to first approximation, the same as the pigment which was photolyzed to produce the intermediates shown, it also contains small contributions due to secondary photoproducts such as the 11-*cis* pigment. In the case of iso-P521 photolysis, this effect probably accounts for the blue shift of the bleach spectrum to 483 nm from the observed λ_{max} of iso-P521, 488 nm. No such shift was observed for the 14-methyl-iso-P521, and the bleach was fit with a Gaussian whose λ_{max} was 490 nm, identical to that of the pigment itself. For the 9-*cis*-α-retinal regenerated sample, much less pigment was available because of poor regeneration yield. This resulted in significantly more noise being present in the time dependent difference spectra, so fewer time points were monitored and more data were collected at those time points. Because of the remaining noise, fitting was confined to the wavelength region where the time-dependent changes were largest. This region is shown in Figure 2 (bottom). Because of the uncertainty in the α-iso-P521 data, the difference between the bleach λ_{max}, 448 nm, and that of the pigment, 455 nm, is probably not significant. Further, no attempt was made to decompose the spectrum of the first species observed after photolysis

Table 2: λ_{max} Values of Intermediates (nm)

species	pigment	Batho	BSI	Lumi	Meta I
gecko P521[a]	521 ± 1	570 ± 3	520 ± 4	516 ± 2	506 ± 2
gecko iso-P521	488 ± 1	560 ± 3	508 ± 4	508 ± 2	503 ± 2
gecko 14-methyl-iso-P521	490 ± 1	560 ± 3	475 ± 4	500 ± 2	502 ± 2
α-iso Gecko P521	455 ± 3		430 ± 3	463 ± 3	455 ± 3
bovine rhodopsin[b]	498 ± 1	529 ± 3	477 ± 3	492 ± 2	
bovine 13-ethyl-isorhodopsin[c]	488 ± 1	539 ± 3	498 ± 4	489 ± 2	
bovine isorhodopsin	485 ± 1	525 ± 3	483 ± 4	487 ± 2	
bovine 14-methyl-isorhodopsin	492 ± 1	545 ± 3	453 ± 4	480 ± 2	

[a] Lewis *et al.* (*7*). [b] Hug *et al.* (*14*). [c] Lewis *et al.* (*11*).

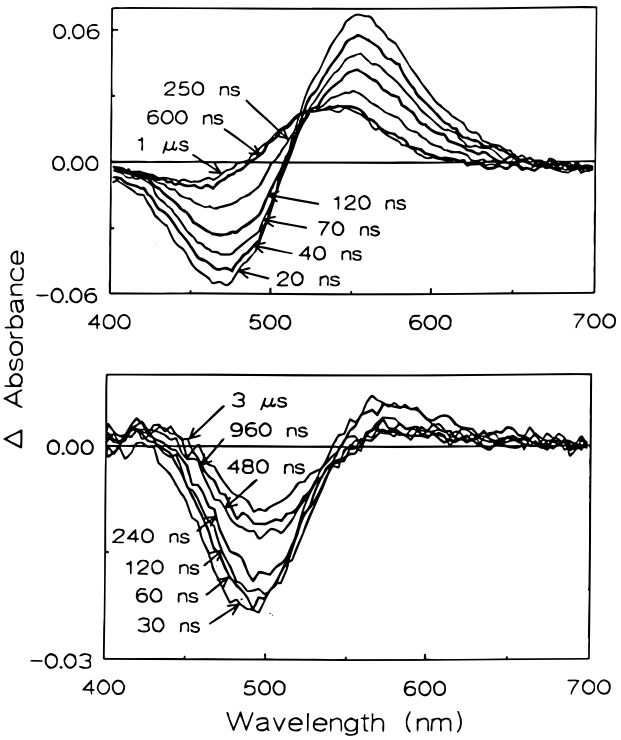


FIGURE 3: Absorbance difference spectra recorded at a series of time delays after 477-nm photolysis of bovine artificial visual pigments in octyl-β-D-glucopyranoside suspensions. (Top) Isorhodopsin. Averaged data from two experiments each using 12 mL of detergent suspension of bovine rhodopsin regenerated with 9-*cis*-retinal. The suspension had an absorbance of 1.0 at 485 nm in the 1.0-cm path length cell used here. (Bottom) Bovine iso-14-methylrhodopsin. Data from an extract of bovine rhodopsin which had been regenerated with 9-*cis*-14-methylretinal. The extract had an absorbance of 0.22 at 492 nm in the 0.2-cm path length cell used here.

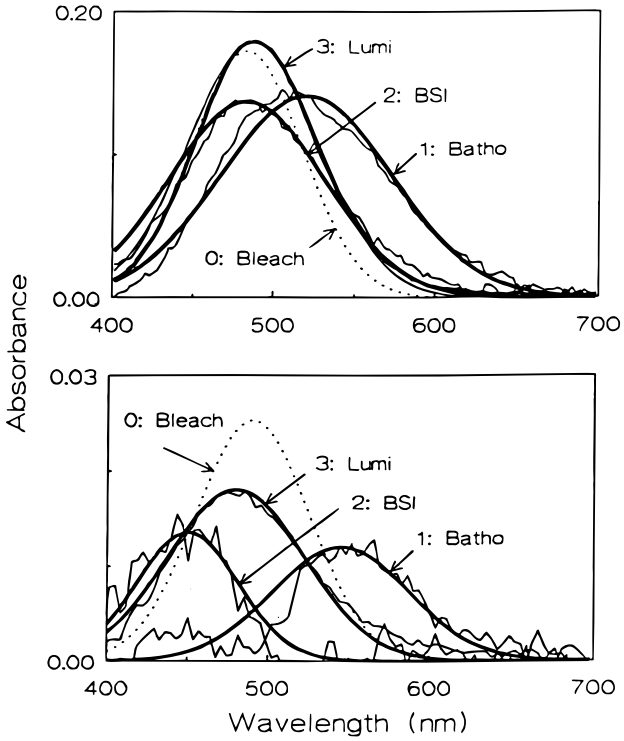


FIGURE 4: Spectra of intermediates observed after photolysis of bovine artificial visual pigments. (Top) Isorhodopsin. Data in Figure 3 (top) were best fit with a biexponential process which corresponds to a sequential decay involving three species with an equilibrium step after the first decay. These can be decomposed using an equilibrium constant of 2.0. This results in the intermediate spectra shown by the light lines. The spectra of the intermediates were fit to Gaussian functions shown by the heavy lines. The λ_{max} for Gaussians is given in Table 2. (Bottom) Bovine iso-14-methylrhodopsin. Spectra for the intermediates computed as described above using an equilibrium constant of 2.0.

into two components, and, for the reasons discussed below, all of its absorbance was assigned to BSI.

Bovine Photolysis Experiments

Data collected after photolysis of bovine isorhodopsin and 14-methyl-isorhodopsin are shown in Figure 3. These data were also best fit by two-exponential processes. However, for the two iso-pigments studied here, as for bovine rhodopsin, these processes correspond to decay of Batho into equilibrium with BSI followed by decay of BSI into Lumi. Detergents such as octyl glucoside perturb bovine Lumi decay, but up to Lumi the kinetics in octyl glucoside are identical to those of native rhodopsin membrane suspensions (*16*); thus only the early intermediates were studied here in these octyl glucoside suspensions. The lifetimes observed for the artificial bovine pigments are given in Table 1. Figure 4 shows the absolute spectra of the intermediates obtained from the global fit by adding back the bleach spectrum. There was evidence for a small amount of conversion of isorhodopsin to rhodopsin, with the isorhodopsin bleach being shifted to 483 nm. There was also a similar shift to 489 nm of the 14-methyl-isorhodopsin bleach observed relative to the 492-nm λ_{max} of the unphotolyzed pigment.

To obtain the absolute intermediate spectrum for BSI, the equilibrium constant for the Batho ⇔ BSI reaction needed to be estimated. The estimate of K_{eq} was based here on simple constraints on the spectra of an acceptable photointermediate, i.e. that it have non-negative absorbance and that its shape be approximately Gaussian. Here a value of 2.0 satisfied these constraints for both isorhodopsin and 14-methyl-isorhodopsin.

Gecko Iso-P521 Chloride Effect

When the iso-P521 sample was dialyzed against chloride-free buffer, the λ_{max} of its absorption difference spectrum shifted to 469 nm. As chloride ion was added back, the visible band intensified, and the λ_{max} red-shifted until it reached 488 nm in 90 mM KCl. In wavelength terms the red shift was approximately 50% complete at a chloride concentration of 1 mM. The data (not shown) were qualitatively similar to results for P521 (*17*) except for a general 33-nm blue shift for the iso-P521 data at comparable chloride concentrations.

DISCUSSION

Iso-P521 Intermediates

Though Tokay gecko retina contains two pigments, P467 and P521, we believe that the P467 contribution to the signals we observe is negligible compared to the noise level of our measurements. Three lines of argument support this conclusion. First, the amount of P467 in gecko retina is at least an order of magnitude smaller than the amount of P521 [our own partial bleaching experiments show ~6% P467 in agreement with Crescitelli (*17*)]. Second, our 477-nm laser source is closer to the 488-nm λ_{max} of iso-P521 than it is to iso-P467, increasing the relative photolysis by the former. Finally, the position of the laser on the blue side of the iso-P521 absorbance band tends to reduce back-photolysis of Batho, increasing the primary photolysis yield, while for iso-P467 the opposite is true.

The difference spectra after photolysis of iso-P521 shown in Figure 1 (top) appear quite different from those observed after similar photolysis of P521 (*7*). This does not necessarily indicate that a different initial photoproduct is formed from the two pigments since the gross form of the difference spectra is determined as much by the photolyzed pigment spectrum as by the photoproduct. The photolyzed pigment contributes to the time independent part of the difference spectra through the bleach while the properties of the initial, thermally unstable photoproduct are contained in the time-dependent part of the difference spectra. When attention is focused on these time-dependent parts, as shown by the results in Table 1, it is clear that the initial photoproducts from P521 and iso-P521 on our time scale are similar if not identical in time dependence. The major difference in the appearance of the data in Figure 1 (top) from the comparable P521 data results from the unusually large blue shift in the λ_{max} of the 9-*cis* cone pigment. For P521 the shift is over twice as large as it is for bovine rhodopsin.

Comparison of the λ_{max} values for the photointermediates in Table 2 shows that at least some of the early intermediates after photolysis of iso-P521 continue to be blue-shifted relative to those from P521. The clearest case is the iso-P521 Lumi intermediate since that intermediate is observed relatively directly. While Batho and BSI suffer from more uncertainty because their spectra are derived from that of the equilibrated mixture, that mixture itself is approximately 15-nm blue-shifted for iso-P521. By choosing somewhat blue-shifted values for the λ_{max}'s of Batho and BSI in Table 2, the K_{eq} for the iso-P521 equilibrated mixture could be made closer to the value of 1 which prevails for P521 itself. If the values in Table 2 were red-shifted, the K_{eq} value would differ even more from the P521 value. It thus seems clear that a significant portion of the extra iso-P521 blue shift persists through the iso-P521 Lumi stage. The blue shift then is much reduced or disappears at Meta I. This can be seen in Figure 2a where the difference between Lumi and Meta I is much smaller than the comparable difference for P521. These data indicate that the extra blue shift of iso-P521 comes from a difference in the protein environment around the chromophore for the 9-*cis* isomer which persists for a few microseconds after photolysis. If the extra blue shift was purely due to a difference in distortion of the 9-*cis* chromophore in P521 relative to bovine rhodopsin, it is likely that the difference would be erased immediately by isomerization. The extra blue shift of iso-P521 does not appear to involve a perturbation of chloride binding. The range of wavelength shift, 20 nm, and apparent binding constant for chloride, 1 mM, that we measured for the iso-P521 sample were similar to those for P521 itself (*4*). In effect the extra iso-P521 blue shift is completely additive with the chloride depletion induced blue shift.

Aside from the persistence of the extra blue shift of the iso-P521 photointermediates, the behavior of iso-P521 after photolysis is very similar to P521. The slight forward shift of the K_{eq} is similar to that observed for bovine isorhodopsin. The somewhat greater amount of secondary photolysis of iso-P521 to P521 compared with bovine isorhodopsin (as evidenced by the larger shift of the bleach spectrum from the pigment λ_{max}) seems consistent with the overlap between the blue-shifted spectrum of the first product which appears during the laser pulse and the wavelength of the actinic light. Greater conversion of iso-P521 to P521 is also favored by the larger red shift of the 11-*cis* pigment compared to bovine rhodopsin.

Iso-14-methyl-P521 Intermediates

The data for iso-14-methyl-P521 shown in Figure 1 (middle) are qualitatively similar to those from iso-P521. However, there are significant quantitative differences between the two pigments resulting from shifts in the λ_{max} values of the intermediates. As shown in Figure 2 (middle), the first species detected has an extremely broad absorption even compared to the iso-P521 case, indicating the presence of a more blue-shifted BSI absorption spectrum. Another difference is that larger changes take place at the later times in the middle part of Figure 1 because of the relatively large difference in the extinction coefficients of the Lumi and Meta I intermediates of iso-14-methyl-P521. Also, some of the difference in the gross form of the data between the top and middle parts of Figure 1 results from the apparently smaller amount of secondary photolysis in the iso-14-methyl-P521 case, again as evidenced by the lack of shift in the bleach relative to the iso-14-methyl-P521 spectrum. The lack of any shift in the bleach must indicate that the 11-*cis* pigment has either a reduced stability or red shift compared to the unmodified retinal chromophore in P521.

The observed rates for decay of the iso-14-methyl-P521 photointermediates are also significantly slowed compared to the iso-P521 data. Some of the slowing of the first observed rate comes from the smaller K_{eq} estimated for iso-14-methyl-P521. However, this does not completely account for the longer first lifetime, and the microscopic decay rate of BSI must also be slower than for the other P521 pigments. That the microscopic rate would decrease for the 14-methyl

substitution is not surprising, since in artificial bovine rhodopsin pigments prepared from other retinal derivatives with added bulk along the polyene chain, the BSI microscopic decay rate has been slowed by a factor up to 4 (*11*). Indeed, as discussed below, this chromophore shows similar slowing of the analogous process in bovine rhodopsin.

α-*Iso-P521 Intermediates*

The α-iso-P521 data are shown in Figure 1 (bottom). Although they are noisy, the data are qualitatively different from those of the other two artificial gecko pigments. As is the case for artificial bovine pigments made with this chromophore (*18*), the Batho product is much less stable relative to BSI. This results in a completely forward shifted Batho ⇔ BSI equilibrium, and consequently we have assigned the spectrum of the first species detected on our time scale to BSI. Because the equilibrium is forward shifted, the first observed rate becomes equal to the microscopic BSI decay rate, 1/60 ns. This is equal within uncertainty to the value calculated for P521, 1/55 ns (*7*).

Bovine Isorhodopsin Intermediates

The time-dependent data for isorhodopsin are very similar to previously published results (*19*), with small differences likely to be due to differences in excitation wavelength and sample temperature. Previous work had not obtained the bleach spectrum, so absolute spectra of the intermediates formed after isorhodopsin photolysis were uncertain. Here the bleach was measured, so the spectra of the intermediates shown in Figure 4 (top) are more precisely determined. As shown in Table 2, small blue shifts are present in the Batho and Lumi spectra for the isorhodopsin intermediates compared to those from rhodopsin, and a small red shift may be present in the spectrum of BSI. The fact that the kinetics are essentially identical for the iso-pigment and for bovine rhodopsin indicates great similarity between the initial photoproducts. Presumably the observed shifts in the intermediate spectra come from distortions of the protein pocket induced by 9-*cis* binding which persist up to the Lumi stage. The situation shows some similarity to the iso-P521 case, but the shifts seen here are smaller and, in the case of BSI from bovine isorhodopsin, opposite to the gecko shift. Little can be concluded from the λ_{max} of bovine isorhodopsin BSI, however, since uncertainty in the K_{eq} makes the λ_{max} of BSI more uncertain than those of Batho and Lumi.

Bovine 14-Methyl-isorhodopsin Intermediates

Figure 3 (bottom) shows the data for 14-methyl-isorhodopsin photolysis, which are quite different from those of isorhodopsin in Figure 3 (top). A major contributor to the differences is the strong absorbance of this pigment relative to its photointermediates. The kinetics also differ substantially from isorhodopsin as shown in Table 1. In particular, the second observed rate has been slowed substantially, a phenomenon which has previously been noted in several other artificial bovine pigments made from retinals with bulk added along the polyene chain. The BSI microscopic decay constant is slowed by a factor of ∼2.5 in 14-methyl-isorhodopsin compared to the case for rhodopsin and is in the range of values from 2 to 4.5 observed for the cases of 9-ethyl-isorhodopsin and 13-ethyl-isorhodopsin (*11*). The spectra of the intermediates (see Figure 4, bottom) are distinguished by an exceedingly large blue shift for BSI (see Table 2). This parallels the case discussed above for iso-14-methyl-P521 except that there the shift was even larger than it is for bovine rhodopsin. Alkyl substitution at C14 is as close as it is possible to make to the PSB, and as such it is likely to exert its effect on either the distance of the PSB from the counterion or alternatively on water molecules near the PSB. The effect on the bovine BSI λ_{max} is opposite for bulk additions at the C14 and C13 positions of retinal (see Table 2). While it would be attractive to assume that at BSI the PSB is shifted closer and farther from the counterion by these substitutions, such a direct effect does not seem consistent with models of PSB/counterion interaction in BSI (*20*). At most, substitutions at these two positions could act to twist the chain oppositely, accounting for their opposite shifts of the BSI λ_{max}, but leaving open the question of why the effect is so much larger for P521 than it is for bovine rhodopsin. Ultimately, the larger blue shifts for iso-P521 and its intermediates relative to bovine rhodopsin may result from P521's extra red shift itself. That it is not easy to construct a pigment with a large opsin shift is demonstrated by the relatively slow progress that has been made in understanding the full structural basis for the red shift of cone pigments. As such, it is possible that cone pigments represent in some sense a more delicately balanced structure and one that reports perturbations with larger blue shifts.

Comparison of P521 and Bovine Iso-Pigments

Previous studies have shown a similar sequence of bleaching intermediates to occur in P521 as occurs after bleaching of bovine rhodopsin (*7, 21*). The results for all the iso-pigments reported here extend that picture of basic similarity in the early processes of these two opsins. Bulk at the 14 position and substitution of 9-*cis*-α-retinal have similar effects in both systems. In the case of iso-14-methyl-P521 the blue shift of the BSI intermediate is even more pronounced than it is for bovine rhodopsin. Also, in both opsins there seems to be some persistence of the blue shifts originally present in the 9-*cis* pigments, at least up to the Lumi stage. These similarities are important for understanding cone pigment photochemistry since more studies of bovine rhodopsin have been conducted. A good deal is known about the red-shifted absorption maximum (opsin shift) of bovine rhodopsin [relative to the retinal protonated Schiff base (PSB) in methanol solution]. Several factors can influence the absorption maximum of the retinal chromophore. Weak interaction between the positively charged Schiff base linkage with its counterion will induce a red shift in the spectrum. Such a red shift can also be induced by increasing double bond conjugations, e.g., by planarization of the ring-chain conformation or of single bonds along the retinal polyene. It was also suggested that interactions with negative charges or polarizable residues can affect the absorption maximum (*22*). It is currently believed that in bovine rhodopsin the red shift is mainly induced by a weak Schiff base/counterion interaction. This was initially difficult to rationalize with the high C═N stretching frequency. However, it was suggested that the counterion is located close to C12 of the retinal (*23,24*) and that one or more water molecules bridge the counterion and PSB (*25*). This picture was supported by model compound studies demonstrating that a red-shifted absorption maximum due to weak counterion−Schiff base interaction is compatible with a high C═N stretching frequency as long as effective hydrogen bonding to the Schiff base proton is maintained (*26*).

With respect to the persistence of the blue shift of the iso-pigments into the early intermediates, iso-P521 shows larger shifts, correlating with the fact that it has more of a blue shift from the 11-*cis* pigment originally. A key observation for understanding the structural basis of these blue shifts is that they are clearly present up to Lumi. The microsecond duration of these blue shifts after photolysis argues strongly against a picture of the iso-pigments in which the 9-*cis* chromophore is distorted to accommodate an essentially undistorted 11-*cis* binding pocket in the opsin. In such a picture the blue shifts might be rationalized as being due to reduced conjugation in the distorted chromophore, with possible contribution from changes in the PSB/counterion distance and orientation. After isomerization, under these conditions, the chromophore would be expected to rapidly achieve a Batho configuration identical to that reached after 11-*cis* photolysis and the blue shift would be expected to disappear within nanoseconds rather than to persist for microseconds as we observe here. Vibrational cooling after excitation typically takes place on the picosecond time scale so that for a blue shift arising solely from a distorted chromophore to persist, bistable equilibrium positions for the relaxed Batho, BSI, and Lumi chromophores would need to be proposed. These seem particularly unlikely since the Batho and BSI intermediates already represent two bistable forms of the relaxed chromophore making further bistable forms less plausible. The persistence we observe thus argues for differences in the iso-pigment pocket from the 11-*cis* configuration and that these differences relax rather slowly after chromophore isomerization.

It is hard to understand how a small perturbation of the protein pocket would persist for microseconds in both these pigments, since the mobility of protein side chains should be fairly high. It therefore seems likely that some significant structural difference exists in the retinal binding pockets of the iso-pigments that produces a systematic blue shift both in the pigments and in the early intermediates. Phenomenologically, the effect is similar to the result of moving the counterion closer to the PSB and this indeed may be the net effect of the modification in the 9-*cis* binding pocket. Change in counterion/PSB interaction could be accomplished directly by displacement of the counterion to a different position in the 9-*cis* pocket or more indirectly by a change in the position of one or more water molecules between the 9- and 11-*cis* versions of the binding pocket. Interpretation in terms of binding site water is more tentative because the interactions of water with the PSB/counterion system are intrinsically more complicated than can be expressed by a simple scalar like PSB to counterion distance. At least one water molecule is believed to occupy the pocket of bovine rhodopsin and, as discussed by Birge (*25*), the difference in the deuterium induced shift in the C═N stretching mode between bovine rhodopsin and isorhodopsin can be interpreted in terms of a water molecule which hydrogen bonds to the PSB with different strengths in these two pigments. It is possible that some difference in the groups to which this water molecule is hydrogen bonded persists in the early intermediates. Previous work (*27*) using low temperature trapping of Batho has shown differences between the spectra of Batho products from bovine rhodopsin and isorhodopsin. That work measured a larger extinction coefficient for Batho produced from isorhodopsin, but did not find the blue shift in Batho λ_{max} seen here at room temperature. In spite of the differences in the specific effects seen on the Batho spectrum, both room temperature and low-temperature studies are consistent with persistent differences between the chromophore environments of bovine rhodopsin and isorhodopsin.

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