Metarhodopsin intermediates of the Gecko cone pigment P521

ARTICLE *in* BIOCHEMISTRY · JANUARY 1994

Impact Factor: 3.02 · DOI: 10.1021/bi00214a018 · Source: PubMed

3 AUTHORS, INCLUDING:

Jie Liang
University of Illinois at Chicago
191 PUBLICATIONS **5,762** CITATIONS

Metarhodopsin Intermediates of the Gecko Cone Pigment P521†

Jie Liang, Rajni Govindjee, and Thomas G. Ebrey*

Biophysics Program and Department of Cell and Structural Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received August 9, 1993; Revised Manuscript Received October 20, 1993®

ABSTRACT: Cone visual pigments are responsible for color vision. Using low-temperature spectroscopy and linear/logarithmic time scale flash photolysis, we studied the photochemistry of the cone visual pigment P521 from the lizard *Gekko gekko*. We found both meta I and meta II intermediates in this cone pigment; meta I absorbs at 480 nm, and meta II absorbs at *ca.* 380 nm. The formation of meta I is a fast process with a lifetime of 2.8 μs for the slow component (pH 6.70, 8.0 °C, 2% digitonin) compared to 2.1 μs for bovine rhodopsin under these conditions. The formation of meta II does not have single-exponential kinetics but can be better characterized by fast and slow components. High pH favors faster kinetics of meta II formation, but less is formed. The amount of meta II formed has a pK_a of 8.7. The fast component of the meta II formation seems to have a somewhat lower pK_a (*e.g.*, 6.4). Temperature also affects meta II formation, with high temperature favoring a faster rate and larger amounts. The higher pK_a of the meta I to meta II transition in gecko P521 compared to a rod pigment like bovine rhodopsin (pK_a = 6.4) probably is due to a cysteine residue at position 211 in gecko rather than a histidine residue in bovine rhodopsin.

In vertebrates, the rod cells in the retina are responsible for scotopic (twilight) vision, while cones are responsible for photopic (daylight) vision. The photochemistry of the scotopic visual pigment rhodopsin has been well studied (Matthews *et al.*, 1963; Ottolenghi, 1980; Parkes & Liebman, 1984; Lewis & Kliger, 1992; Ebrey & Liang, 1993). Upon absorbing light, the 11-*cis*-retinal chromophore of rhodopsin is isomerized to the *all-trans* conformation. The activated pigment then goes through a series of photochemical changes before meta I ($\lambda_{max} \approx 480$ nm) decays to meta II ($\lambda_{max} \approx 380$ nm). Meta II activates the GTP binding protein transducin, which in turn activates a cGMP phosphodiesterase, lowering the cGMP concentration. As a result, cGMP-regulated ion channels in the plasma membrane are closed and the rod hyperpolarized (Stryer, 1986; Pugh & Cobbs, 1986). In bovine rhodopsin, meta I and meta II are in a pH-dependent equilibrium with a pK_a of 6.4; meta II then decays slowly to meta III or directly to free retinal and opsin (Matthews *et al.*, 1963; Blazynski & Ostroy, 1984).

In contrast, the activating photochemical processes in photopic core visual pigments are still unknown. So far, there is only a brief report on a meta II like intermediate of chicken iodopsin (Hubbard *et al.*, 1965; Yoshizawa *et al.*, 1991). It is important to understand the photochemical reactions of such photopic pigments to understand the functioning of cones and their differences with rods. Here we report the photochemical transformations of the cone pigment from the gecko retina.

The photoreceptors from retinas of Tokay gecko (*Gekko gekko*) are an excellent source for a cone pigment. There are two visual pigments, P467 and P521, in the retina. The amino acid sequence of P467 is similar to other rod visual pigments sequenced. The amino acid sequence of P521, on the other hand, is similar to the long-wavelength-absorbing cone pigments responsible for color vision (Kojima *et al.*, 1992). Moreover, not only does P521 have a spectral location (λ_{max} = 521 nm) close to several cone pigments such as the human green cone pigment (λ_{max} = 531 nm) but also it has many other characteristics shared by long-wavelength cone pigments. For example, the λ_{max} of both P521 and the chicken red-sensitive cone visual pigment can be shifted to the blue by removing chloride ions. They both can be bleached by NH_2-OH in dark. They both regenerate quickly when their apoprotein is mixed with 11-*cis*-retinal. P467 and the rhodopsins have none of these properties (Crescitelli, 1977, 1992; Crescitelli & Liu, 1988).

One advantage of working with the Tokay gecko system is the abundance of P521 in the retina. P521 accounts for about 90% of the total visual pigment, with P467 making up the other 10% (Crescitelli, 1977). Another advantage of Tokay gecko retina is that it does not contain the oil droplets often found in cone cells, which simplifies pigment purification (Crescitelli, 1977).

Crescitelli (1977) noticed that the P521 pigment has no detectable meta III intermediate. Here we are able to identify the meta I and meta II intermediates of the gecko P521 pigment. We found that gecko P521 forms an intermediate analogous to meta I of bovine rhodopsin upon light excitation, which then decays to a meta II like intermediate. These two intermediates are in a pH-dependent equilibrium, with a pK_a of *ca.* 8.7.

MATERIALS AND METHODS

Dissection. Tokay geckos were dark-adapted for 8 h. All subsequent operations were carried out under dim red light. The geckos were put in a 4 °C cold room for about 30 min to slow their activity so they could be handled easily. A sharp needle was then used to double-pith them. After decapitation, the gecko eyeballs were excised and hemisected with a sharp razor blade, and the retina was removed using a pair of forceps and immediately put into a beaker at 4 °C containing a pH 7.40 solution of 20 mM MOPS buffer, 100 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol.

Rod Outer Segment Preparation. Gecko rods were prepared according to methods for preparation of bovine rod outer

† This work was supported by NIH Grant EY01323 to T.G.E.

* To whom correspondence should be addressed at the Department of Cell and Structural Biology, University of Illinois at Urbana–Champaign.

® Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/93/0432-14187$04.00/0

segments (Jonas and Ebrey, unpublished results). The purified gecko rod outer segment fragments were suspended in 100 mM KCl solution with or without 5 mM $MgCl_2$.

Visual Pigment Preparation. The rod fragments were extracted overnight at 4 °C with 2% digitonin in 100 mM KCl prepared according to Bridges (1977). Various concentrated pH buffer solutions were then added to aliquots of the pigment extract to attain a 10 or 20 mM final buffer concentration. All the data presented here were obtained from pigment samples in buffered digitonin solutions.

Flash Photolysis. Flash photolysis was carried out on pigment samples of different pHs at set temperatures with a kinetic spectrophotometer as described previously (Govindjee *et al.*, 1988). The sample path length was 2 mm. The activating light was a 532-nm, 7-ns pulse from a Nd-YAG laser (Quanta Ray DCR).

In some measurements, a logarithmic timebase, as described by Austin *et al.* (1975), was used to acquire the transient absorbance signal. Data collection in logarithmic measurements started 2.5 μs after the laser flash. The data were stored in blocks of 25 data points. The duration of each block lasts twice as long as that of the preceding one. Any of the 25 data points in the nth data block was an average of 2^n sampling points, whereas all sampling points were acquired at a uniform interval of 1 μs from the measuring system. The averaging was accomplished by adding all the sampling points within the $2^n \times 1$ μs period and dividing by the number of samples 2^n in this period. In all logarithmic time scale experiments, 3-mm path-length cuvettes were used.

Low-Temperature Spectra. Absorption measurements at low temperatures were carried out using an Aviv 14-DS spectrophotometer with a home-made dewar, as described in Balashov *et al.* (1991). Digitonin extracts of the gecko pigment were mixed with glycerol (1:1) for these experiments. To bleach the sample, a 500-W projector was used with an optical light guide and a glass cutoff filter (Corning 3-67; transmitted $\lambda > 550$ nm).

Optical Multichannel Analyzer Spectra (OMA). Light-induced difference spectra of the intermediates after 532-nm laser activation were obtained by measuring the absorbance changes at various time intervals from 50 μs to 500 ms with an optical multichannel analyzer system (Model IRY512G; Princeton Instruments Corp., Princeton, NJ).

Titration Model. All titration data were fitted by the Henderson–Hasselbalch equation using either a home-made Marquadt–Levenberg nonlinear least-squares curve-fitting program written in Pascal or the curve-fitting function of KaleidaGraph (Synergy software) on a Macintosh to determine the pK_a and the number of protons involved, n.

RESULTS

Identification of the Photointermediates Meta I and Meta II from Gecko P521. We studied the absorption changes at 380 and 460 nm after selective excitation of the cone pigment P521 with a 532-nm laser pulse. All changes are due to photochemical reactions of P521 and its intermediates, since the other pigment present, P467, is only 10% of the total pigment and its absorption maximum is far from the excitation laser wavelength of 532 nm such that little of the pigment is activated. Figure 1 shows the absorbance changes of P521 in 10 mM MOPS buffer of pH 6.64 at 12.5 °C. At 460 nm, a transitory intermediate (tentatively identified as the analogue of meta I from its time course and λ_{max}, see below) is formed in less than 1 ms, and it then decays into some other intermediate. The absorbance changes at 380 nm indicate

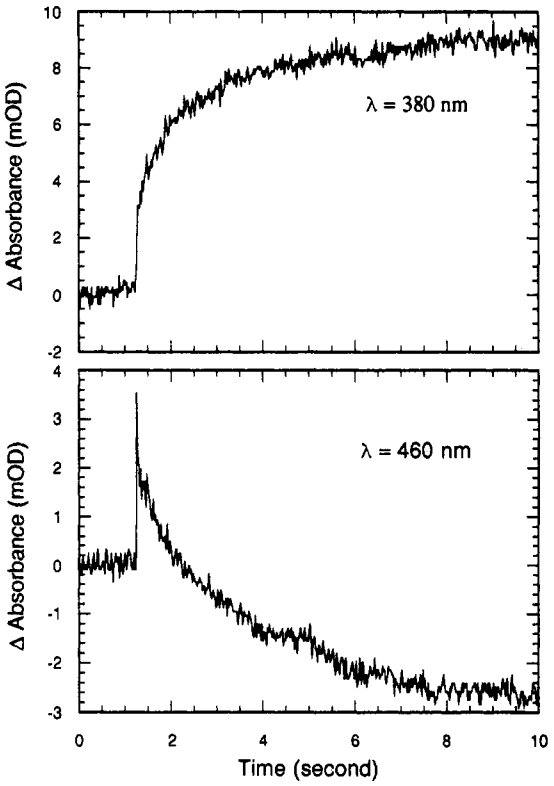

FIGURE 1: Absorbance changes of the gecko cone pigment P521 at 380 and 460 nm after 532-nm Nd-YAG laser excitation. Sample in 2% digitonin, 100 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS buffer (pH 6.64) at 12.5 °C.

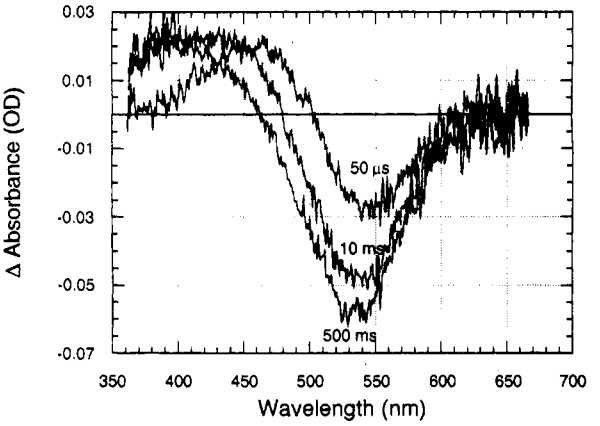

FIGURE 2: Difference spectrum of P521 recorded with an optical multichannel analyzer (OMA) 50 μs, 10 ms, and 500 ms after 532-nm laser excitation. Samples were in 2% digitonin with 100 mM KCl, 5 mM $MgCl_2$, and 10 mM PIPES buffer at pH 6.5, 22 °C.

that a species absorbing in the ultraviolet is formed in a time scale comparable with that of the decay of the species at 460 nm. This is presumably the meta II intermediate.

Flash-induced difference spectra of P521 were measured with an optical multichannel analyzer at a series of different time intervals. Figure 2 shows spectral changes of P521 at pH 6.5 (10 mM PIPES buffer), room temperature (22 °C), 50 μs, 10 ms, and 500 ms after the flash. At 50 μs, an absorbance change at *ca.* 460 nm is observed in the difference spectra due to the formation of meta I. From 10 ms on, more and more meta II is formed around 390 nm.

We also measured the laser-induced absorbance changes of P521 (pH 6.07 in 10 mM MES, 8.0 °C) every 10 nm from 360 to 570 nm (except for 530 nm, which is too close to the laser wavelength). The intermediate that formed 1 ms after

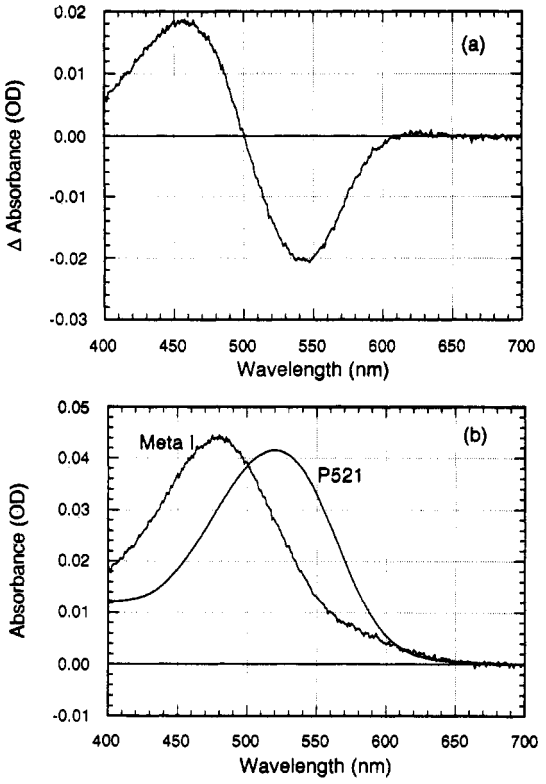

FIGURE 3: (a) Light-induced difference spectrum of P521 at –40 °C. Irradiated for 20 min with a 500-W projector through a light-guide and a 550-nm cutoff filter. (b) P521 spectrum at room temperature and reconstructed meta I spectrum at –40 °C.

the bleach has a maximum at 460 nm in the difference spectrum. At 10 ms, the absorbance at 460 nm partly disappears, and a new species with a λ_{max} of about 400 nm in the difference spectrum, presumably the meta II intermediate, starts to appear. These results were obtained in an independent method, and they confirm the pattern of the P521 absorbance change from OMA on Figure 2.

Meta I Intermediate. P521/MI difference spectra were also obtained at low temperature. Spectra of pigment samples at –40 °C were taken before and after 20 min of bleaching with a long-wavelength cutoff filter (Corning glass filter 3-67, transmitted $\lambda > 550$ nm). The spectrum after irradiation is a mixture of the remaining P521 pigment and the newly formed meta I, and perhaps some 9-*cis* pigment, since it is possible that the extended period of bleaching may convert some batho intermediate into the 9-*cis* isomer of P521. Figure 3a shows the bleaching difference spectrum due to formation of meta I and bleaching of P521. The minimum is at 540 nm, the isosbestic point at 500 nm, and the maximum at 455 nm.

The low-temperature difference spectrum correlated well with the OMA difference spectrum at 50 μs (Figure 2) and the flash-induced wavelength-by-wavelength difference spectrum 1 ms after bleaching. This shows that after light excitation the newly formed intermediate meta I can be trapped in –40 °C. To reconstruct the meta I absorption spectrum, we assume that meta I does not absorb substantially at wavelengths longer than 580 nm, and that only a small amount of 9-*cis* pigment is present, not enough to affect the spectrum significantly. The spectrum of an appropriate amount of P521 can be then added to the difference spectrum to obtain the spectrum of meta I (Figure 3b). The P521 spectrum was obtained by taking the spectrum of the digitonin extract of the gecko retina and removing the 10% contribution due to P467 absorbance. The correction for P467 is achieved by subtracting a simulated P467 spectrum derived from blue-

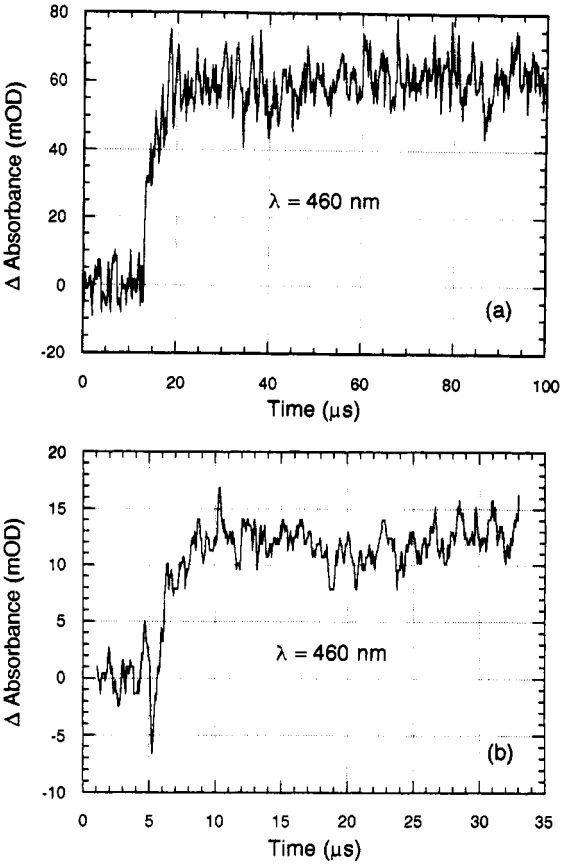

FIGURE 4: (a) Kinetic trace of the formation of the gecko meta I intermediate monitored at 460 nm in 100 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS buffer at pH 6.70, 8.0 °C. The lifetime of the slow component of meta I formation is 2.8 μs. (b) Bovine meta I formation monitored at 460 nm in 1% digitonin, 100 mM KCl, and 20 mM MOPS buffer at pH 6.47, 9.5 °C. The lifetime of the slow component of meta I formation for bovine is *ca.* 2.1 μs.

shifted octopus rhodopsin (original λ_{max} = 475 nm), since no reliable P467 spectrum could be obtained. This correction is small, and errors in it should not greatly distort the meta I spectrum. From the reconstructed absorption spectrum shown in Figure 3b, the λ_{max} of gecko meta I is 480 nm.

There are several possible sources of error for the above method of reconstruction of the meta I intermediate spectrum from the bleaching difference spectrum. For example, the existence of a 9-*cis* iso-pigment, and possible change in the spectrum upon cooling, as well as the amount of P521 added back to the bleaching difference spectrum would all introduce errors in the λ_{max} estimation. We estimate that the λ_{max} for the meta I spectrum in Figure 3b is accurate within ±6 nm.

The kinetics of the formation of meta I measured at 460 nm, pH 6.70, and 8.0 °C are shown in Figure 4a. An initial fast component (kinetically unresolved) is followed by a slower component which has an estimated lifetime, τ, of 2.8 μs. The kinetic pattern is comparable with that of bovine rhodopsin under similar conditions, which has a τ of *ca.* 2.1 μs estimated for the slow component, as shown in Figure 4b.

Meta II Formation. Upon raising the temperature after –40 °C irradiation, meta I decayed to an intermediate absorbing in the ultraviolet. This is the meta II intermediate. Figure 5 shows the difference spectra obtained between an irradiated sample at –34 °C which is then warmed to temperatures between –30 and –3 °C. We can see the disappearance of meta I at *ca.* 480 nm with the concurrent formation of meta II at *ca.* 380 nm. This is consistent with the OMA difference spectra in Figure 2 and the

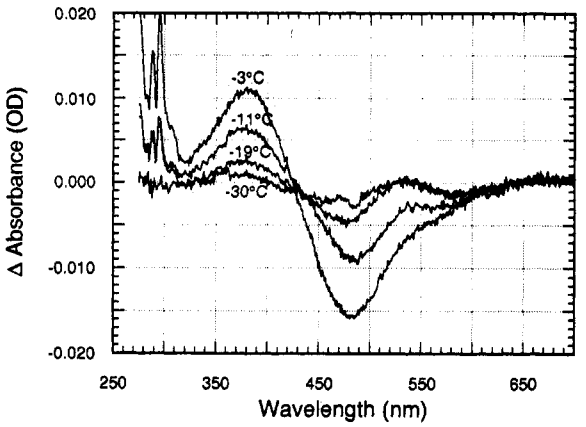

FIGURE 5: Difference spectra of irradiated P521 upon raising the temperature from −34 °C to the temperatures indicated. Meta I disappears, and a short-wavelength intermediate, meta II, appears.

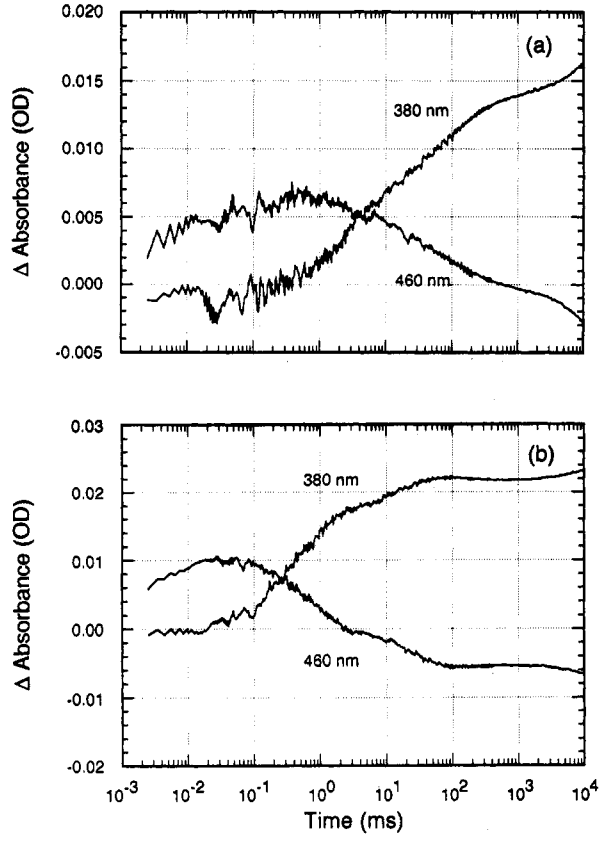

FIGURE 6: (a) Kinetics of the flash-induced absorbance changes of the gecko cone pigment P521 at 380 and 460 nm with a logarithmic time scale. The samples were in 2% digitonin, 100 mM KCl, and 20 mM MES buffer at pH 5.80, 26 °C. (b) Kinetic changes of bovine rhodopsin at 380 and 460 nm with a logarithmic time scale. The samples were in 2% digitonin, 100 mM KCl, and 20 mM MOPS, pH 7.21 at 26 °C.

wavelength-by-wavelength flash difference spectrum 10 ms after the bleaching.

Unfortunately, we are unable to construct a reliable meta II absorbance spectrum using a method similar to that used for the meta I spectrum, because of an unexplained absorbance change at *ca.* 580 nm when the temperature was raised and because of the propagated error.

The kinetic absorbance changes of gecko cone pigment P521 at 26 °C are shown in Figure 6a using a logarithmic time scale. The absorbance change caused by the 380-nm monitoring beam alone is negligible: after 5 s of recording, the monitoring beam caused only about 4% of the signal. For 460-nm measurements, the monitoring beam bleached less than 0.5% of the sample after 5 s. Since the total 460-nm signal after 5 s is small (about 1.5% of the absorbance of the sample), bleaching introduced a larger relative error for the 460-nm signal than for the 380-nm signal.

For comparison, the changes of bovine rhodopsin in 2% digitonin, pH 7.21, 26 °C are shown in Figure 6b. The amount of meta I (460 nm) reaches a plateau in about 15 μs. There is an increase in absorbance at 380 nm and decrease at 460 nm from *ca.* 30 μs to *ca.* 80 ms, corresponding to the transition of bovine MI (478 nm) to MII (380 nm). From *ca.* 80 ms to *ca.* 600 ms, there is a very small absorbance decrease at 380 nm and a concurrent small increase at 460 nm. This probably reflects the transition of a tiny portion of the bleached pigment from meta II (380 nm) to meta III (465 nm). Starting from about 2 s, the absorbance increases at 380 nm and decreases at 460 nm, which may be a combination of free retinal formation (from MII and/or MIII) and monitoring beam bleaching. The latter has a larger effect with 460-nm measurements.

In digitonin solutions of gecko cone pigment P521, the absorbance changes in Figure 6a show an increase at 380 nm occurring from *ca.* 200 μs to *ca.* 600 ms (pH 5.80, 100 mM KCl, 20 mM MES, 26 °C). This increase at 380 nm corresponds to the concurrent decrease at 460 nm. Then a plateau with a slight increase at 380 nm and a decrease at 460 nm is maintained from *ca.* 600 ms to *ca.* 2.5 s. In contrast to bovine rhodopsin (Figure 6b), there is no change that can be attributed to the meta II to meta III transition. The lack of a meta III intermediate confirms Crescitelli's result that meta III is absent when no nitrate was added (Crescitelli, 1977).

Effects of pH on Meta I and Meta II Intermediates of P521. Figure 7a shows several flash-induced kinetic absorbance traces at 380 nm of gecko P521 for various pHs on a logarithmic time scale (20 °C). These changes reflect the formation of meta II and free retinal. At our experimental temperature of 20 °C, we assume that meta II formation will be complete within 10 s, and the absorbance change at 380 nm that extends beyond 10 s reflects formation of free retinal. At pH 5.44, meta II formation starts from *ca.* 1 ms. However, the meta I to meta II transition cannot be described with a single exponential over the more than 3 decades of time recorded. A curve-fit with two-exponents shows that the fast component has a τ of 8.5 ms and the slow component has a τ of 300 ms. After 1 s, meta II formation is slower and approaches a plateau, indicating the establishment of equilibrium between meta I and meta II. At pH 7.17, the fast phase (until *ca.* 150 ms) and the slow phase (from *ca.* 150 ms) are all present, but the plateau disappears. Instead, a continued increase at 380 nm is seen at about 2 s, and continued beyond 10 s. This may indicate that the formation of the free retinal from meta II at pH 7.17 starts before the completion of the establishment of the equilibrium between meta I and meta II. At pH 8.49, the general kinetic features look similar to those at pH 7.17, except the free retinal formation starts a little earlier. At pH 9.04, only a small amount of meta II is formed. The slow component of meta II formation is much smaller, and a plateau appears again; *i.e.*, an equilibrium between meta I and meta II is reached at about 60 ms, well before the onset of free retinal formation. When the pH is raised to 9.87, the amount of meta II formed is negligible.

It is interesting to note that even though the amount of meta II formed is very small at high pH (*e.g.*, pH 9.87), free retinal is still formed at 10 s, and is likely to continue to

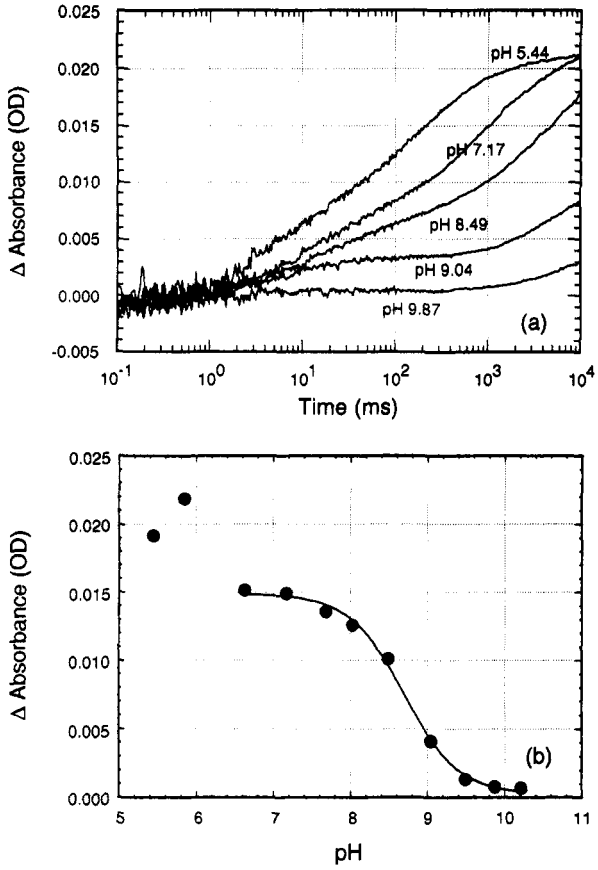

FIGURE 7: (a) Flash-induced kinetic absorbance changes of gecko cone P521 at 380 nm on a logarithmic time scale. The P521 sample was in 2% digitonin with 100 mM KCl and 20 mM buffer of pH 5.44 (MES), 7.17 (MOPS), 8.49 (BICINE), 9.04 (CHES), or 9.87 (CAPS). The temperature was 20 °C. (b) pH dependence of gecko P521 meta II formation measured 1 s after a 532-nm flash. The samples were at 20 °C in 2% digitonin, 100 mM KCl, and 20 mM buffer of pH 5.44 (MES), 5.85 (MES), 6.63 (ADA), 7.17 (MOPS), 7.68 (MOPS), 8.02 (HEPPS), 8.49 (BICINE), 9.04 (CHES), 9.49 (CHES), 9.87 (CAPS), or 10.21 (CAPS).

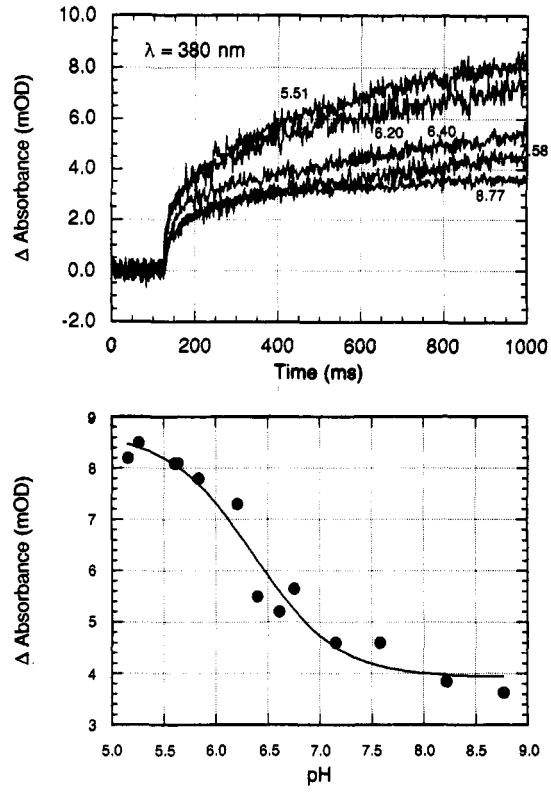

FIGURE 8: (a) Flash-induced kinetic absorbance changes at 380 nm of gecko cone P521, 8.0 °C. The samples were in 2% digitonin with 100 mM KCl, 5 mM $MgCl_2$, and 10 mM buffer of pH 5.51 (MES), 6.20 (MES), 6.40 (MES), 7.58 (HEPES), or 8.77 (CHES). (b) Amount of meta II formed 1 s after 532-nm excitation at various pHs. The pK_a is 6.36 with an n of 1.1.

accumulate afterward. This is probably due to the equilibrium between the meta I and meta II intermediates. As small amounts of meta II slowly decay to free retinal, new meta II is formed from meta I to maintain the pH-dependent equilibrium between meta I and meta II. Thus, large amounts of free retinal accumulate from meta I, flowing through a small steady concentration of meta II.

In general, the overall features of the meta II formation can be outlined as the following: at low pH, meta II formation is complete by around 2 s. At higher pH, the kinetics of formation of both meta II and free retinal are accelerated. However, the amount of meta II formed is smaller when the pH is high. At very high pH (*e.g.*, 9.04), meta II formation can reach an equilibrium with meta I before free retinal formation starts.

From the above analysis of the kinetic features of meta II formation, we decided that 1 s is a good time point to use, since most of the meta II is formed at 20 °C regardless of pH, yet the interfering effect of free retinal formation is absent when the pH is low, or negligible compared with meta II formation when the pH is higher. A plot of the amount of meta II formed at 1 s *vs* pH will give a good approximation of the pH dependency of the meta I to meta II transition. Figure 7b shows that when data above pH 6.5 were fitted, the meta I to meta II transition has a pK_a of 8.7 at 20 °C. Roughly one proton is involved in this process.

One way to study the initial fast component is to slow the process down by lowering the temperature. The absorbance changes at 380 nm 1 s after the flash at 8 °C will have a larger contribution from the initial component than at 20 °C. Figure 8a shows absorbance changes at 8 °C for several pH values *vs* time. The meta I–meta II equilibrium formed under these conditions has a pK_a of 6.4 (Figure 8b). Since the rod pigment P467 consists of only 10% of the total pigment, and the excitation wavelength 532 nm is far from P467's λ_{max}, P467's contribution is negligible. Although it is arguable whether this method reflects most accurately the characteristics of the initial component, we can draw qualitative conclusions from this experiment. Apparently, the initial component of meta II formation has a different pH dependency which is characterized with a lower pK_a (6.4) than that of the overall meta II formation at/near the equilibrium stage (pK_a 8.7). Note that at a high pH, such as 8.77, there still is some meta II formed (3.6 mOD out of 8.2 mOD at pH 5.15). This presumably can be attributed to the early effect of the slow component of the meta I to meta II transition which has a higher pK_a.

Effects of Temperature on Meta I and Meta II Intermediates of P521. Temperature also has a strong influence on the kinetics of gecko P521 meta II formation. In these studies, we used MOPS (10 mM, pH 6.64 at 20 °C) as the buffer, since it has a small temperature coefficient (−0.0006/°C). The pH change of MOPS buffer over a 30 °C span is no greater than 0.2 pH unit. Figure 9 shows the kinetics of the meta I to meta II transition on a 5-s time scale. From these 5-s traces, we can see that the kinetic rate increased significantly when the temperature was raised from 8.0 to

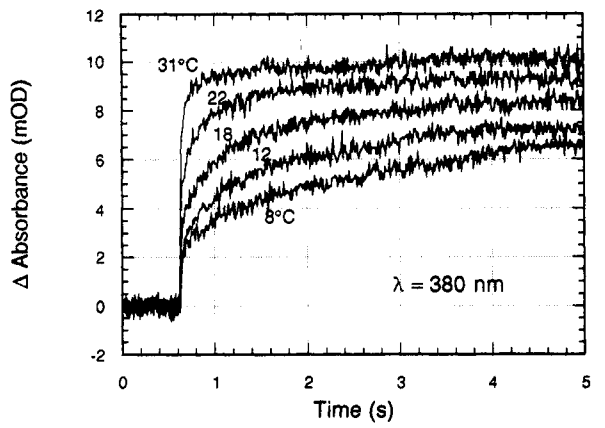

FIGURE 9: Effect of temperature on the meta I to meta II transition at 380 nm. The samples were in 100 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS buffer, pH 6.64 and 20 °C.

30.7 °C. The amount of meta II formed at the end of the 5-s period also increased as the temperature increased.

DISCUSSION

Although many papers have dealt with the meta I and meta II intermediates in rhodopsin, very little is known about the corresponding photochemical changes in cone pigments. To study the photochemistry of cone pigments, we used the gecko P521 pigment, which has strong amino acid sequence similarity to other long-wavelength absorbing cone pigments (Kojima *et al.*, 1992). Our results showed that the intermediates analogous to meta I and meta II in bovine rhodopsin can be clearly seen in this cone pigment from optical multichannel analyzer, single-wavelength flash photolysis, and low-temperature spectroscopic studies. The spectrum of meta I at −40 °C has a λ_{max} of 480 nm. The λ_{max} of meta II is estimated to be *ca.* 380 nm. Unlike bovine rhodopsin, we do not see a detectable meta III intermediate in our kinetic studies. Preliminary low temperature bleaching experiments (−170 °C) on P521 suggest the existence of a batho-like intermediate for gecko cone pigment, with an estimated λ_{max} of *ca.* 555 nm (data not shown).

The formation of the meta I intermediate for P521 in digitonin is found to be a fast process with a lifetime of 2.8 μs for the slow component at 8.0 °C, pH 6.70, in digitonin. At 20 °C, the establishment of the meta I–meta II equilibrium is almost complete in 5 s at pH 5.44, but is accelerated when the pH is raised to 9.04, where the transition is completed within 200 ms. The kinetic characteristics of this transition cannot be described by a single exponential. A plausible approximation is to describe the process with two components. For example, at pH 5.44, the meta I to meta II transition can be described by a fast component with a τ of 8.5 ms and a slow component with a τ of 300 ms.

pH not only affects the kinetic rate of the meta I to meta II transition but also affects the final amount of meta II formed. In digitonin, we found that the pK_a for meta II formation is about 8.7, with a slope of $n = 1.2$. However, the initial component seems to have a different pH dependency than the overall formation of meta II, with a lower pK_a estimated as *ca.* 6.4.

It has been suggested that the histidine-211 residue of rhodopsin is mainly responsible for the pH dependence of the meta I to meta II transition (Weitz & Nathans, 1992). Gecko P521 lacks such a histidine residue. Instead, it has a cysteine residue at the corresponding position (Cys-211) (Kojima *et al.*, 1992). This cysteine is conserved in all known long-wavelength cone pigments (Tang and Ebrey, unpublished observations). Unlike the bovine mutants H211C and H211F, gecko cone pigment P521 does form a meta II intermediate; the pK_a between meta I and meta II is high, 8.7. This suggests that the cysteine in the long-wavelength absorbing cone pigment can also serve as the functional group in lieu of the histidine residue. The pK_a of 8.7 at equilibrium seems to correspond well with such a hypothesis, since cysteine is known to have a pK_a of *ca.* 8.7 in solution. It is intriguing that at 2 °C the bovine rhodopsin mutants H211C and H211F form little meta II at either pH 5.8 or pH 7.8, yet at 25 °C pH 6.4, large amounts of meta II and free retinal are formed (Weitz & Nathans, 1992). It is possible that at different temperatures, different components of the meta I to meta II transition dominate, and these components may have a different pH dependency, as we suggest in the case of gecko cone P521.

On the other hand, bovine rhodopsin has two other histidine residues (H65 and H152) which seem to increase the pK_a of the meta I and meta II equilibrium, in contrast to H211 which lowers the pK_a. Weitz and Nathans suggested that these additional histidine residues are responsible for the shallow slope of the meta I and meta II equilibrium ($n < 1$) found in bovine rhodopsin (Weitz & Nathans, 1992). Our results support their suggestion: gecko P521 lacks these two additional histidine residues, and indeed the equilibrium meta I to meta II titration has a steeper curve that has an $n > 1$. This suggests that the titration curve for the meta I to meta II equilibrium in other cone pigments such as iodopsin may also have a steep slope, similar to what we found in gecko, since iodopsin lacks these histidine residues as well. However, we find that the kinetics of the meta I to meta II transition cannot be accurately described with one component. This suggests that the combinatorial states of protonation of these two additional histidine cannot entirely account for the existence of multiforms of meta I and meta II intermediates in bovine rhodopsin.

In general, cones have lower photosensitivities and more rapid electrophysiological kinetics than rods. Cones need about 100 times more photons to reach a half-maximal signal than rods (Baylor *et al.*, 1979). The photocurrent of both mammal and amphibian cones rises faster and is terminated more rapidly than rods (Normann & Werblin, 1974). One hypothesis about the low photosensitivity for exciting cones is that the cone meta II intermediate is not formed efficiently or in enough quantity. Our results do not support such a hypothesis, since the pK_a of the meta I and meta II equilibrium we found in P521 is 8.7, larger rather than smaller to what was found in bovine (pK_a 6.4). This suggests that physiological pH conditions do not hinder meta II formation in cone pigments. The low photosensitivity of cone pigments must be due to factors other than cone pigment photochemistry.

It has been shown that in spite of the predominance of the cone pigment, gecko photoreceptors actually respond relatively slowly to the onset and termination of light excitation (Toyoda, 1969). With respect to its electrophysiological response, the gecko rod is like typical rod-pigment containing rods. This suggests that whether a pigment is cone- or rod-like has little to do with whether the photocurrent response of the photoreceptor is a fast cone type or a slow rod type. Indeed, our experiments showed that the kinetics of meta II formation of gecko cone pigment P521 are actually slower than those of bovine rhodopsin (see Figure 6a,b). Since gecko cone P521 has a high degree of amino acid residue sequence similarity with other long-wavelength-absorbing cone pigments, we believe that the photochemistry of gecko P521 exemplifies that of other long-wavelength-absorbing cone pigments. This

suggests a lack of correlation between the photochemistry of the pigment (cone type or rod type) and the overall photocurrent responses (fast cone type or slow rod type).

ACKNOWLEDGMENT

We are grateful to the Frauenfelder group and Burr Nelson for developing the logarithmic data acquisition system and software, Masahiro Kono for helping in kinetic measurements, and Li Tang for preparation of bovine rods. We thank Professor Mudi Sheves and Dr. Jim Lewis for reading our manuscript.

REFERENCES

Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Gunsalus, I. C. (1975) *Biochemistry 14*, 5355–5373.

Balashov, S. P., Govindjee, R., & Ebrey, T. G. (1991) *Biophys. J. 60*, 475–490.

Baylor, D. A., Lamb, T. D., & Yau, K.-W. (1979) *J. Physiol. 288*, 613–634.

Blazynski, C., & Ostroy, S. E. (1984) *Vision Res. 24*, 459–470.

Bridges, C. D. B (1977) *Vision Res. 17*, 301–302.

Crescitelli, F. (1977) in *Handbook of Sensory Physiology* (Crescitelli, F., Ed.) Vol. VII/5, pp 391–449, Springer-Verlag, Heidelberg.

Crescitelli, F. (1992) *Prog. Retinal Res. 11*, 2–32.

Crescitelli, F., & Liu, R. S. H. (1988) *Proc. R. Soc. London, B 233*, 55–76.

Ebrey, T. G., & Liang, J. (1993) in *CRC Handbook of Organic Photochemistry and Photobiology* (Song, P.-S., Ed.) CRC Press, Boca Raton, FL (in press).

Govindjee, R., Dancshazy, Zs., Ebrey, T. G., Longstaff, C., & Rando, R. R. (1988) *Photochem. Photobiol. 48*, 493–496.

Hubbard, R., Bownds, D., & Yoshizawa, T. (1965) *Cold Spring Harbor Symp. Quant. Biol. 30*, 301–315.

Kojima, D., Okano, T., Fukada, Y., Shichida, Y., Yoshizawa, T., & Ebrey, T. G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6841–6845.

Lewis, J. W., & Kliger, D. S. (1992) *J. Bioenerg. Biomembr. 24*, 201–210.

Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.

Normann, R. A., & Werblin, F. S. (1974) *J. Gen. Physiol. 63*, 37–61.

Ottolenghi, M. (1980) *Adv. Photochem. 12*, 97–200.

Parkes, J. H., & Liebmann, P. A. (1984) *Biochemistry 23*, 5054–5061.

Pugh, E. N., & Cobbs, W. H. (1986) *Vision Res. 26*, 1613–1643.

Stryer, L. (1986) *Annu. Rev. Neurosci. 9*, 87–119.

Toyoda, J.-I., Nosaki, H., & Tomita, T. (1969) *Vision Res. 9*, 453–463.

Weitz, C. J., & Nathans, J. (1992) *Neuron 8*, 465–472.

Yoshizawa, T., Shichida, Y., & Fukada, Y. (1991) *Pure Appl. Chem. 63*, 171–176.