

## The $pK_a$ of the Protonated Schiff Bases of Gecko Cone and Octopus Visual Pigments

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**ABSTRACT** A visual pigment is composed of retinal bound to its apoprotein by a protonated Schiff base linkage. Light isomerizes the chromophore and eventually causes the deprotonation of this Schiff base linkage at the meta II stage of the bleaching cycle. The meta II intermediate of the visual pigment is the active form of the pigment that binds to and activates the G protein transducin, starting the visual cascade. The deprotonation of the Schiff base is mandatory for the formation of meta II intermediate. We studied the proton binding affinity,  $pK_a$ , of the Schiff base of both octopus rhodopsin and the gecko cone pigment P521 by spectral titration. Several fluorinated retinal analogs have strong electron withdrawing character around the Schiff base region and lower the Schiff base  $pK_a$  in model compounds. We regenerated octopus and gecko visual pigments with these fluorinated and other retinal analogs. Experiments on these artificial pigments showed that the spectral changes seen upon raising the pH indeed reflected the  $pK_a$  of the Schiff base and not the denaturation of the pigment or the deprotonation of some other group in the pigment. The Schiff base  $pK_a$  is 10.4 for octopus rhodopsin and 9.9 for the gecko cone pigment. We also showed that although the removal of  $Cl^-$  ions causes considerable blue-shift in the gecko cone pigment P521, it affects the Schiff base  $pK_a$  very little, indicating that the  $\lambda_{max}$  of visual pigment and its Schiff base  $pK_a$  are not tightly coupled.

### INTRODUCTION

Visual pigments consist of 11-*cis* retinal (Stryer, 1986) covalently bound to an apoprotein via a protonated Schiff base linkage. The  $pK_a$  of the protonated Schiff base in the pigment is many pH units higher than that for protonated Schiff base model compounds. Light initiates a series of changes in the pigment (the "bleaching intermediates"), which eventually leads to the activation of the GTP-binding protein transducin, which controls a cGMP phosphodiesterase; the cGMP in turn controls the cation permeability of the plasma membrane of the photoreceptor cell whose change generates electrophysiological signal. The meta II bleaching intermediate is responsible for the activation of transducin (Koutalos and Ebrey, 1986; Stryer, 1986). Meta II's formation is accompanied by the deprotonation of the Schiff base linkage (Doukas et al., 1978); without such deprotonation, there is no activation of transducin (Longstaff et al., 1986). Understanding of the magnitude and control of the  $pK_a$  of the Schiff base deprotonation is thus crucial in clarifying the mechanism of visual excitation.

The absorption spectrum of bovine rhodopsin does not change much as the pH is raised to pH 11, indicating a very high Schiff base  $pK_a$  (Lythgoe, 1937; Wald, 1938; Koutalos, 1992). Recent studies using artificial bovine rhodopsin pigments suggest that the Schiff base  $pK_a$  of bovine rhodopsin is above 16 (Steinberg et al., 1993). Upon raising the pH of

octopus rhodopsin, the spectrum is blue-shifted by about 100 nm from 475 nm with a  $pK_a$  of 10.6 (Koutalos et al., 1990), suggesting that octopus rhodopsin's Schiff base can be deprotonated with a much lower  $pK_a$  than bovine rhodopsins. In the gecko cone pigment P521, we report here a similar spectral blue-shift from 521 to 375 nm, with or without  $Cl^-$  ions, when the pH is raised. This again suggests that we are titrating the protonated Schiff base. Alternatively, the increased pH could be just denaturing the octopus and gecko pigments, which would expose the Schiff base-binding site to the bulk solvent, leading to its deprotonation. If so, the apparent  $pK_a$  is just that for the denaturation of the protein. A related explanation for the observed  $pK_a$  is that another group of the pigment is being titrated and that its deprotonation results in exposure of the Schiff base to solution.

One way to test whether these pH-dependent absorption changes are associated directly with the titration of the Schiff base is to regenerate artificial visual pigments with retinal analogs that have electron-withdrawing fluorine groups attached to the polyene. In model compounds, 13- $CF_3$  retinal lowers the Schiff base  $pK_a$  from 7.4 to 1.8. A similar  $pK_a$  decrease is observed in bacteriorhodopsin after regeneration with the 13- $CF_3$  retinal analog (Sheves et al., 1986), indicating that the protonated Schiff base  $pK_a$  of bR can be controlled by such fluorinated retinal analogs. 14-F retinal is another retinal analog bearing a strong electron withdrawing group. In solution, the 14-F substitution lowers the  $pK_a$  of the protonated Schiff base model compound by  $\sim 2.4$  units relative to the protonated Schiff base of native retinal (Steinberg et al., 1993).

In this work, we studied octopus rhodopsin and the gecko cone pigment P521. As noted above, the absorption spectra of both pigments show blue-shifts when the pH is raised,

Received for publication 3 February 1994 and in final form 16 May 1994.

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0006-3495/94/08/848/07 \$2.00

suggesting that their Schiff bases are becoming deprotonated. We found that the 9-*cis* 14-F retinal regenerated artificial octopus pigment has a pK<sub>a</sub> that is 3.5 units lower than that of the 9-*cis* pigment, indicating that the titration observed in the native octopus system is indeed associated with titration of the Schiff base. The spectral change observed in the gecko cone pigment at high pH is likely also to be associated with the titration of Schiff-base linkage, because 14-F substitution in the retinal lowers the pK<sub>a</sub> of this transformation by about 1.1 pH units.

## MATERIAL AND METHODS

### Chromophores

The retinal chromophores used are shown in Fig. 1: (1) 11-*cis* retinal, as found in native visual pigments, (2) 9-*cis* retinal, (3) 9-*cis* 14-F retinal, (4) 9-*cis* α-retinal, (5) 9-*cis* 14-methyl-retinal, and (6) 9-*cis* 9-desmethyl-retinal. Chromophore 3 was prepared as previously described (Asato et al., 1978), 4 according to Arnaboldi et al., (1979), 5 according to Chan et al., (1974), and 6 according to Blatz et al., (1969).

### Membrane preparations

Octopus (*Paroctopus delilei*) microvilli were prepared according to Koutalos et al. (1989). Gecko retinal outer segments were prepared according to Liang et al. (1993).

### Bleaching

A previously described protocol was followed for bleaching the visual pigments (Koutalos et al., 1989). Photoreceptor membranes from either octopus or gecko were suspended in a 38% sucrose solution in 100 mM KCl buffer (20 mM MOPS, pH 7.2 for gecko; 20 mM MES, pH 6.0 for octopus) with 0.2 M of hydroxylamine. The bleaching was carried out in an ice-cold bath in an initially dark room, using light of wavelength longer than 480 nm for octopus and 530 nm for gecko (Corning cutoff filters 2–70 and 3–68, respectively). With a 400-W projector, bleaching was complete in less than 15 min for gecko and 60 min for octopus. The membranes were then washed 4 times at 4°C (JA-20 rotor in a J2–21 Beckman centrifuge, at 20K rpm for

30 min) with MOPS (gecko) or MES (octopus) buffer to remove the hydroxylamine, and the pellet was suspended in 38% sucrose in the same buffer used for the washes. This was used for regeneration with retinal analogs.

### Regeneration

Two equivalents of the chromophore was dissolved in ethanol (final volume was less than 1% of that of the apomembrane solution), and mixed with the apomembrane. The spectra were recorded before and after addition of chromophores. All regenerations were performed either at room temperature for 3 h or at 4°C overnight. Regenerated pigments were washed at least once with 100 mM KCl.

### Spectral titration

The spectra were recorded with an Aviv 14DS equipped with an end-on photomultiplier. Each regenerated membrane sample, 2 ml in a quartz cuvette, was magnetically stirred throughout the titrations to prevent sedimentation. A baseline spectrum of the same cuvette, with 2 ml of distilled water, was subtracted from all spectra. The pH was raised by adding 5 μl KOH of various normality. Each spectrum was corrected for dilution. The pH was recorded with a combination electrode (Beckman Instruments (Fullerton, CA) or Radiometer America (Westlake, OH)). Titrations of gecko outer segments in their Cl<sup>-</sup>-deficient state were carried out without directly measuring pH, because the leaking of the Cl<sup>-</sup> from the pH electrode would destroy the Cl<sup>-</sup> deficient state. Therefore, the pH was measured using an identical sample solution that was prepared at the same time. A few minutes was usually enough for equilibration, as judged by stabilization of the pH meter's reading, and then the pH value was recorded. All titrations were carried out at room temperature of 23°C.

### Titration model

All titration data were fitted by the Henderson-Hasselbach equation using either a home-made Marquadt-Levenberg nonlinear least-square curve-fitting program or the curve-fitting function of KaleidaGraph software (Synergy software) on Macintosh to determine the pK<sub>a</sub> and the number of protons involved.

## RESULTS

### Spectral titration of 9-*cis* and 9-*cis* 14-F-regenerated octopus microvilli

Upon raising the pH from ~8.0, the spectrum of octopus rhodopsin shifts ~100 nm to the blue, indicating the deprotonation of the Schiff base. This spectral shift has an intrinsic pK<sub>a</sub> of 10.6 (Koutalos et al., 1990). The native pigment has 11-*cis* retinal (1) as its chromophore. After regeneration of bleached octopus rhodopsin in microvilli with 9-*cis* retinal (2), this artificial octopus pigment can also be titrated by monitoring the blue shift of the spectrum from 460 to 366 nm. Fig. 2 shows an example of the pH titration of the 9-*cis* octopus pigment in 4 M KCl. Fig. 2 A shows the changes in the absorbance spectra with pH and Fig. 2 B shows the difference spectra. The pK<sub>a</sub> of this transition, ~10.4 (Fig. 4), is similar to the native 11-*cis* pigment's pK<sub>a</sub> of 10.6 at the same salt concentration.

Fig. 3 shows the titration of the spectral blue shift of the artificial pigment formed from bleached octopus photoreceptor microvilli regenerated with 9-*cis* 14-F-retinal (3). Fig. 3 A shows the absorbance spectra changes in 4 M KCl;

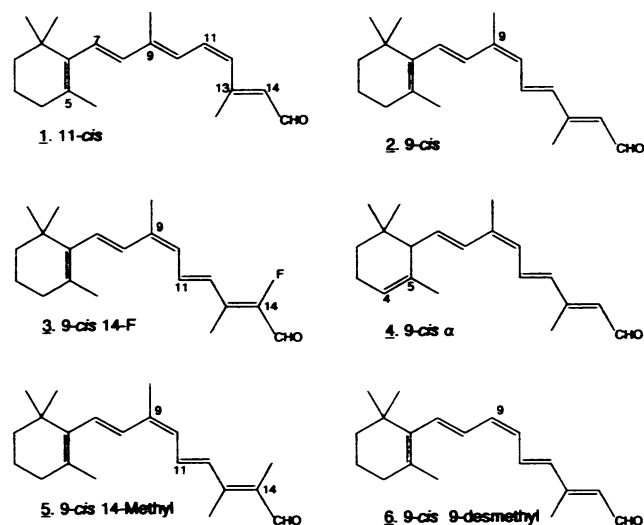


FIGURE 1 The structures of retinal and its analogs: (1) 11-*cis*, (2) 9-*cis*, (3) 9-*cis* 14-F, (4) α-9-*cis*, (5) 9-*cis* 14-methyl, (6) 9-*cis* 9-desmethyl retinal.

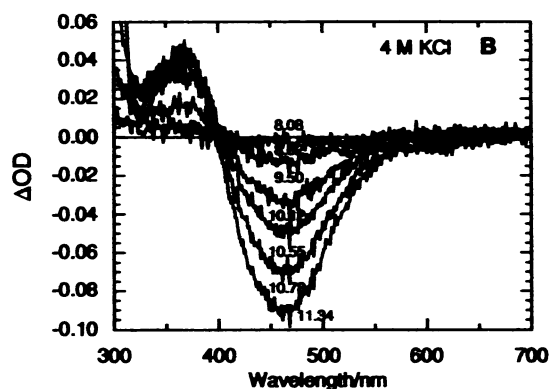
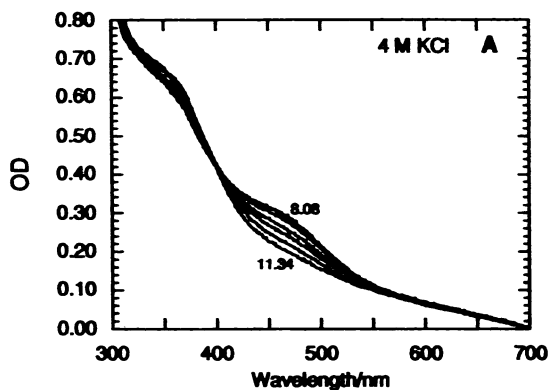


FIGURE 2 The pH titration of the spectrum of 9-*cis* octopus rhodopsin in microvillar membranes in 4.0 M KCl. (A) absorbance and (B) difference spectra.

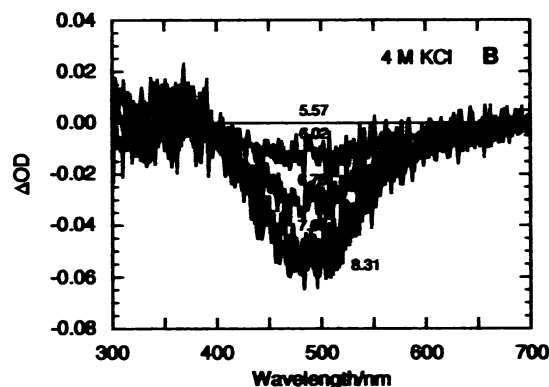
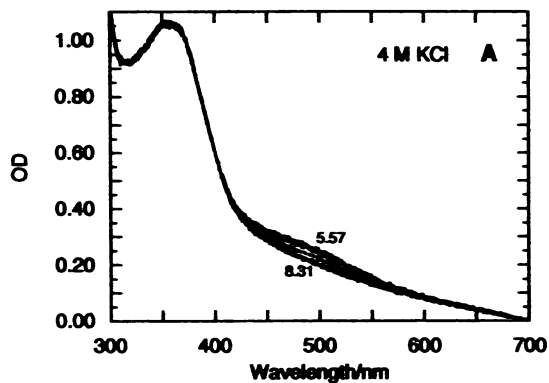


FIGURE 3 The pH titration of the spectrum of 9-*cis* 14-F octopus rhodopsin in microvillar membranes in 4.0 M KCl. (A) absorbance and (B) difference spectra.

Fig. 3 B shows the titration difference spectra. For 9-*cis* 14-F octopus microvilli, the  $pK_a$  of the spectral titration is  $\sim 6.8$  (Fig. 4).

The titration curves from several experiments with 9-*cis* (2) and 9-*cis* 14-F (3) retinal-regenerated octopus pigments in 4.0 M KCl are shown in Fig. 4. The difference of  $\sim 2.4$  pH units between 9-*cis* and 9-*cis* 14-F-protonated retinal Schiff base model compounds in solution (Steinberg et al., 1993) is reflected in the difference in the artificial pigments'  $pK_a$  values of 3.5 pH units. This result demonstrates that the spectral change in octopus that occurs after pH elevation is indeed associated with the deprotonation of the protonated Schiff base.

### Spectral changes of the gecko cone pigment P521 with increasing pH

There are two visual pigments in the retina of the *Gekko gekko*: the P521 cone pigment and the P467 rod pigment, with the cone pigment dominating (about 90% of the visual pigments (Crescitelli, 1977; Kojima et al., 1992)). Crescitelli first noticed that the spectrum of gecko cone pigment is blue-shifted upon raising the pH (Crescitelli, 1992). Here we report a full titration of gecko photoreceptor membrane in 50 mM KCl. Fig. 5 A shows the absorbance spectral change, and

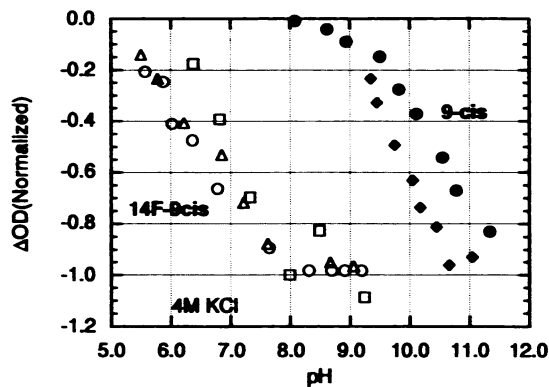


FIGURE 4 The pH titrations of the 9-*cis* and 9-*cis* 14-F retinal regenerated octopus pigments in 4.0 M KCl and 50 mM KCl. Different symbols represent independent experiments.

Fig. 5 B shows the difference spectra. As the absorbance at 521 nm decreases, a concurrent slight increase at  $\sim 380$  nm is present. The  $pK_a$  of this spectral change is 9.9.

It is possible that such a spectral change induced by high pH could be caused by simple protein denaturation, which would be unlikely to be reversible. We found that gecko P521 visual pigment does not seem to be denatured by these pHs.

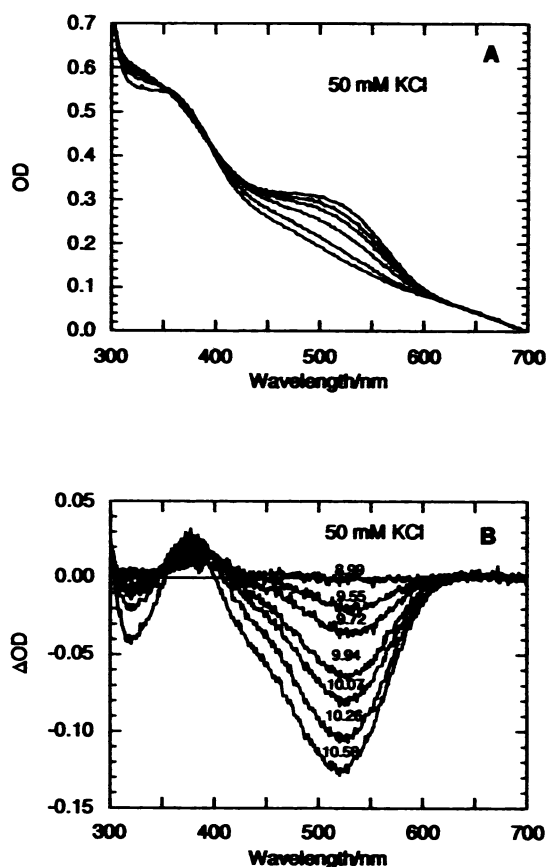


FIGURE 5 Effect of pH on the absorption spectrum of native gecko visual pigment in outer segments in 50 mM KCl. (A) absorbance and (B) difference spectra.

Two identical aliquots of gecko outer segments in 50 mM KCl were used for a complete pH titration and for the reversibility test. The complete pH titration gave the maximum extent of the titration. To test reversibility, the pH of the second sample was first raised to 9.9, followed by HCl addition to lower the pH and to attempt to reverse the spectral change. When the pH was lowered to 8.5, 72% of the spectral change was recovered. Although protein denaturation is likely to occur when the pH is raised to very high values, e.g., above 10, we conclude that the spectral changes seen at lower pHs are mainly caused either by direct titration of the pigment's protonated Schiff base or by titration of some other group that causes the exposure of the Schiff base. We tested this second possibility as well as protein denaturation by the use of the fluorinated chromophores which intrinsically lowered the  $pK_a$  of the protonated Schiff base.

#### Titration of artificial gecko pigment derived from 9-cis and 9-cis 14-F retinals

The spectral changes induced by raising the pH were recorded for 9-cis and 9-cis 14-F retinal-regenerated artificial gecko pigments. Fig. 6 shows the difference spectra of 9-cis (Fig. 6A) and 14-F, 9-cis (Fig. 6B) artificial gecko pigment membrane titrations. For the 9-cis and 9-cis 14-F pigments

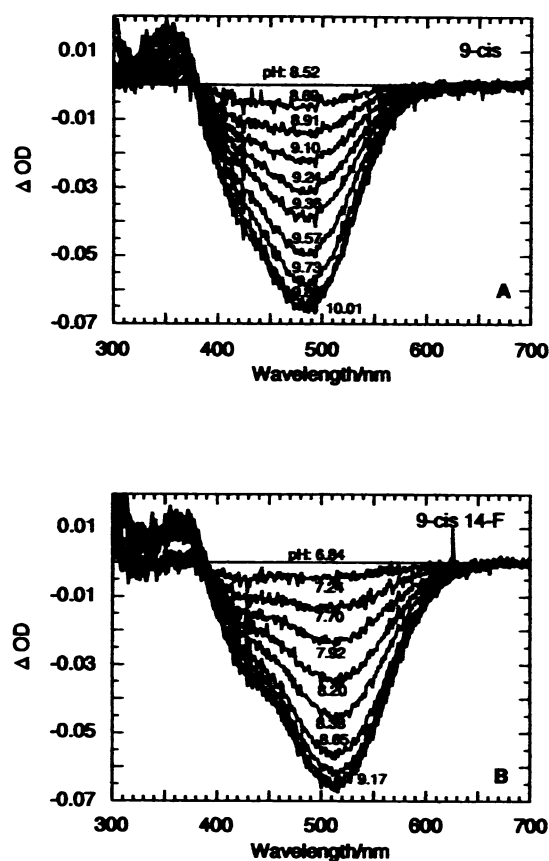


FIGURE 6 pH difference spectra of (A) 9-cis and (B) 9-cis 14-F-regenerated gecko visual pigments.

in 50 mM KCl, the spectral titrations are characterized by  $pK_a$  values of  $\sim 9.3$  and  $\sim 8.2$ , respectively (Fig. 7). Note the absorbance spectrum of the 9-cis pigment at pH 8.0 is not used as baseline in Fig. 6A, because an absorbance change at 420 nm occurs between pH 8.0 and 8.5, presumably because of the titration of random Schiff bases formed during regeneration. We estimated the absorbance change at  $\sim 485$  nm of the 9-cis artificial pigment between pH 8.0 and 8.5, and included the data in Fig. 7.

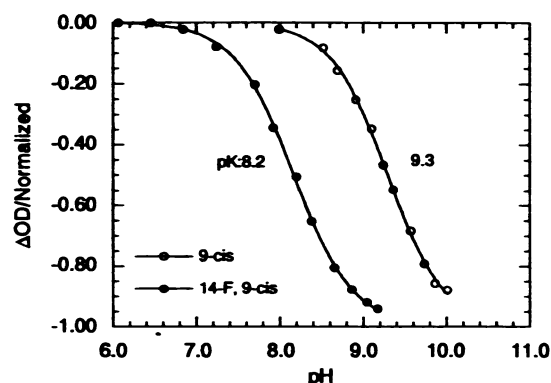


FIGURE 7 pH titration  $pK_a$  of 9-cis and 9-cis 14-F-regenerated gecko pigments in outer segments in 50 mM KCl.

## Titration of other artificial gecko pigments

### 9-cis 14-methyl artificial gecko pigment

To test if the change in the  $pK_a$  of the visual pigment upon introduction of the 14-F group noted above is caused by a steric perturbation of the chromophore caused by the 14-F group, we tested the titration behavior of the 9-cis 14-methyl (5) regenerated artificial gecko pigment. This retinal analog lacks the electron withdrawing fluorine group, but bears a methyl group that could sterically affect the pigment in a similar way as the fluorine would, albeit the methyl group is bulkier than fluorine.

In 50 mM KCl, 9-cis 14-methyl retinal regenerated gecko pigment titrates with a  $pK_a$  of 9.6 (see Fig. 8), about 0.3 pH units higher than that of the 9-cis-regenerated gecko pigment. In solution, the addition of the 14-methyl group to retinal lowers the  $pK_a$  of the protonated Schiff base in model compound by  $\sim 0.4$  units (M. Sheves, unpublished observations). This suggests that the steric perturbation of 14-methyl within the protein binding site raises the  $pK_a$  by a total of  $\sim 0.7$  units if the data of 14-methyl model compound is incorporated (0.4 units to compensate  $pK_a$  drop observed in model compound and 0.3 units for the apparent  $pK_a$  increase in the pigment). This experiment indicates that the steric perturbation alone of the 14-substitution would increase the  $pK_a$ , not decrease it.

### 9-cis $\alpha$ -retinal artificial gecko pigment

9-cis  $\alpha$ -retinal (4) differs from 9-cis retinal only in the ring region with shortened conjugated double bonds. In model compounds, it has no effect on the protonated Schiff base  $pK_a$  (M. Sheves, unpublished observations). Fig. 8 shows that in 50 mM KCl, gecko cone pigment regenerated with 9-cis  $\alpha$ -retinal has a  $pK_a$  of 8.7, about 0.5 pH units lower than that of the 9-cis artificial pigment. This result suggests that a perturbation in the ring region has an effect on the environment of the Schiff base region, which results in a moderately lowered  $pK_a$ .

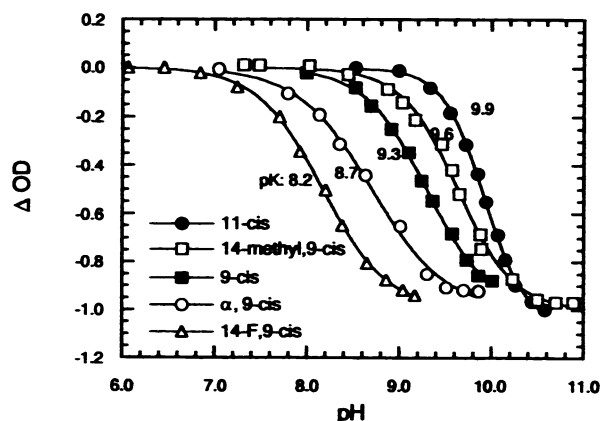


FIGURE 8 pH titration of 11-cis (native), 9-cis-14-methyl, 9-cis,  $\alpha$ -9-cis, and 9-cis 14-F containing gecko visual pigments in 50 mM KCl.

### Other artificial gecko pigments

The 9-cis, 9-desmethyl retinal (6)-regenerated visual pigment has a very unusual property: in bovine rhodopsin, this artificial pigment has no detectable meta II intermediate (Ganter et al., 1989). It would be interesting to find out whether the underlying cause is a change in the protonated Schiff base  $pK_a$ . In this study, we have checked the influence of removing the 9-Me group on the  $pK_a$  of the gecko pigment. pH titration of 9-cis 9-desmethyl regenerated gecko cone pigment showed that its  $pK_a$  is about 9.6, only about 0.3 units higher than that of 9-cis artificial pigment. This indicates that the effect on the  $pK_a$  of 9-desmethylation is not significant in the gecko pigment.

9-cis 13- $CF_3$  and 9-cis 12,13-dichloro retinals also bear strong electron withdrawing groups near the Schiff base. Unfortunately, these retinal analogs failed to regenerate with bleached gecko apomembrane.

### The $Cl^-$ -deficient state does not have a different Schiff base $pK_a$

The absorption spectrum of the gecko cone pigment P521, like other long wavelength absorbing visual pigments, is dependent on the presence of  $Cl^-$ . When  $Cl^-$  is absent from the solution, the  $\lambda_{max}$  of P521 is blue-shifted to 497 nm (Crescitelli, 1977), and the extinction coefficient is reduced. An intriguing possibility is that the  $pK_a$  of the spectral titration is affected as well when  $Cl^-$  is absent, and when the  $\lambda_{max}$  of the visual pigment is altered.

P521-containing membranes were suspended in 50 mM  $K_2SO_4$  with or without 5 mM KCl. The difference in ionic strength by the addition of 5 mM KCl is small, and 5 mM KCl saturates the  $Cl^-$ -binding site of iodopsin, the best characterized long wavelength-absorbing cone pigments (Kleinschmidt and Harosi, 1992), and probably will also saturate that of P521.

Fig. 9 shows the pH titration of gecko P521 in its normal and  $Cl^-$ -deficient states. Because the extinction coefficient of P521 in the  $Cl^-$ -deficient state is smaller, the total change

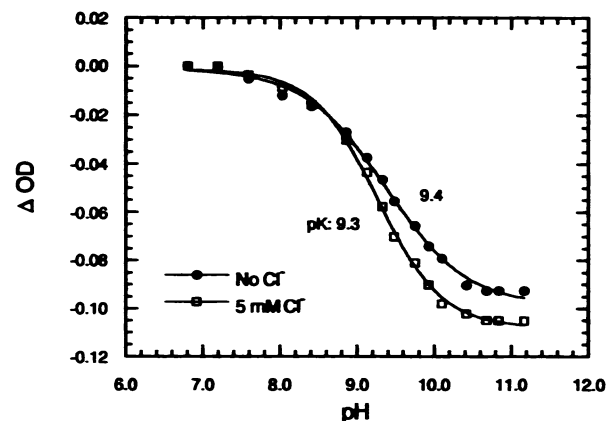


FIGURE 9 Titration  $pK_a$  plots of native gecko outer segments in 50 mM  $K_2SO_4$  with and without 5 mM KCl.

of absorbance is also smaller in Cl<sup>-</sup>-deficient state (Fig. 9). The pK<sub>a</sub> of pH titration of Cl<sup>-</sup>-deficient state in 50 mM K<sub>2</sub>SO<sub>4</sub> is 9.4, very close to that of the Cl<sup>-</sup>-sufficient state of 9.3 in 50 mM K<sub>2</sub>SO<sub>4</sub> and 5 mM KCl. The change in absorption λ<sub>max</sub> caused by Cl<sup>-</sup> depletion is rather large, 24 nm, but the pK<sub>a</sub> of the two forms of the pigment are the same. Thus, these results suggest that the absorption spectrum and Schiff base pK<sub>a</sub> are not necessarily correlated.

## DISCUSSION

Table 1 summarizes experimental results of Schiff base titrations of octopus rhodopsin and gecko cone visual pigment P521.

### Octopus

Our pH titrations of octopus pigments indicated that the pK<sub>a</sub> of the large blue-shift in the spectrum is lowered from ~10.4 for 9-*cis*-regenerated artificial pigment to ~6.8 for 9-*cis* 14-F-regenerated artificial pigment. This difference of 3.6 pH units is even greater than the value (2.2 pH units) found for model 9-*cis* and 9-*cis* 14-F-protonated retinal Schiff bases in solution. This is a strong evidence that the spectral change caused by raising the pH is indeed associated with the deprotonation of the Schiff base of octopus rhodopsin.

Although the changes in the pK<sub>a</sub> of the protonated Schiff base by 14-F retinal in bacteriorhodopsin (Sheves et al., 1986) and in sensory rhodopsin (Spudich, personal communication) are very close to those found in their respective model compounds, in octopus there is a difference between the pK<sub>a</sub> of its visual pigment and that of the model protonated Schiff base. Such a discrepancy is not unexpected, because the environment in the protein is different from what prevails in solution. This change can contribute to the pK<sub>a</sub> and alter the substituent electrostatic effect observed in solution.

### Gecko P521 pH-dependent change

The absorption spectrum of gecko cone pigment P521 also significantly blue-shifts upon raising the pH. However, P521 differs from octopus rhodopsin in several respects. It has a Cl<sup>-</sup>-dependent absorption spectrum, and the pK<sub>a</sub> of its spectral titrations are different for 9-*cis*-regenerated (pK<sub>a</sub> = 9.3) and the native 11-*cis* (pK<sub>a</sub> = 9.9) pigments. Both of these differences indicate that the retinal binding sites in octopus rhodopsin and gecko cone P521 are different.

In our studies, we found that 9-*cis* 14-F retinal can lower the pK<sub>a</sub> of the spectral titration of the P521 artificial pigment by about 1.1 pH units. The pK<sub>a</sub> of 9-*cis* retinal-based P521 is raised by ~0.3 pH units by the 14-methyl group, showing that the steric perturbation induced by introducing an additional group at position 14 tends to increase the pK<sub>a</sub>. Considering that 14 methyl lowers the pK<sub>a</sub> in model compound by 0.4 units, the actual pK<sub>a</sub> increase from steric effect of 14-methyl group within the binding site is ~0.7 units. The real decrease of pK<sub>a</sub> in 14-F P521 can thus be deduced to be higher than 1.1 units, as the combination of the part that compensates the steric perturbation increase and the observed net 1.1-unit decrease. This is close to the 2.4-unit change found for model 9-*cis* and 9-*cis* 14-F-protonated Schiff base in solution. As a result, it is likely that the pK<sub>a</sub> lowering effect of 14-F substituent is caused by its electron withdrawing capability, not a steric effect.

Other changes in the retinal moiety also affect the pK<sub>a</sub> of the Schiff base of analogs. 9-*cis* α-retinal-regenerated P521 has a shortened conjugated double-bond system for its retinal, and carbon 6 has an sp<sup>3</sup> rather than the sp<sup>2</sup> character found in the native system. This alteration can induce a perturbation because of steric reasons as well. We note that in bacteriorhodopsin incorporation of a similar retinal analog (5,6-dihydro-retinal) into the binding site changes the C=N stretching frequency (Schiffmiller et al., 1985), and in bovine rhodopsin the photochemistry of the artificial pigment derived from α-retinal is significantly altered (Randall et al., 1991). We found that in gecko pigment this chromophore lowers the spectral titration pK<sub>a</sub> by about 0.5 units. 9-desmethyl-retinal regenerated P521 also showed a different Schiff base pK<sub>a</sub>: its pK<sub>a</sub> is raised about 0.3 units compared with that of 9-*cis* regenerated artificial pigment. These retinal analogs, together with that of 9-*cis* 14-methyl, are all characterized by steric perturbations of the retinal binding site. The changes in pK<sub>a</sub> are all moderate (0.3–0.5 pH-unit change). 9-*cis* 14-F retinal can cause a larger change in the spectral titration pK<sub>a</sub> (1.1 units, and higher if the steric effect in the opposite direction is counted), indicating that the mechanism of the pK<sub>a</sub> change for this retinal analog is different from that of the other analogs, i.e., it is an effect of the electron-withdrawing property of fluorinated retinal on the Schiff base linkage. From the above analysis, we suggest that

TABLE 1 Titrations of protonated Schiff base of native and artificial visual pigments

Pigment	Retinal	[Salt]	λ <sub>max</sub> of PSB	λ <sub>max</sub> of SB	pK <sub>a</sub>
Octopus*	11- <i>cis</i>	4 M KCl	475 nm	376 nm	10.6
Octopus	9- <i>cis</i>	4 M KCl	468 nm	362 nm	10.4
Octopus	9- <i>cis</i> 14-F	4 M KCl	~498 nm	~370 nm	6.8
Gecko P521	11- <i>cis</i>	50 mM KCl	521 nm	376 nm	9.9
Gecko P521	9- <i>cis</i>	50 mM KCl	487 nm	350 nm	9.3
Gecko P521	9- <i>cis</i> 14-F	50 mM KCl	517 nm	~365 nm	8.2
Gecko P521	9- <i>cis</i> 14-Met	50 mM KCl	496 nm	360 nm	9.6
Gecko P521	9- <i>cis</i> α	50 mM KCl	461 nm	~350 nm	8.7
Gecko P521	9- <i>cis</i> 9-desmethyl	50 mM KCl	~450 nm	~360 nm	9.6
Gecko P521	11- <i>cis</i>	50 mM K <sub>2</sub> SO <sub>4</sub>	~495 nm	~370 nm	9.4
Gecko P521	11- <i>cis</i>	50 mM K <sub>2</sub> SO <sub>4</sub> + 5 mM KCl	521 nm	370 nm	9.3

\* From Koutalos et al. (1990).

the pH-induced spectral change in P521 is also directly associated with the deprotonation of the Schiff base.

Recently it was suggested that the  $pK_a$  of the retinal-protonated Schiff base and its counterion in retinal proteins is controlled by a defined angle between the protonated Schiff base linkage and its counterion, which allows water to bridge the two groups and to stabilize the ion pair (Gat and Sheves, 1993). The steric perturbation enforced by the retinal analogs described above might induce a change in the orientation of the donor and acceptor groups, thereby reducing the stabilization energy introduced by the water molecules. This could provide an explanation for the steric effects on the  $pK_a$  of the Schiff base (Gat and Sheves, 1993).

### Cl<sup>-</sup>-dependent changes

Kleinschmidt and Harosi (1992) proposed a model for the effect of Cl<sup>-</sup> on the absorption spectrum of iodopsin in which the Schiff base environment consists of a counterion interacting with the protonated Schiff base through a bound Cl<sup>-</sup> and several structural water molecules. In the absence of Cl<sup>-</sup>, the complex structure collapses and the counterion interacts directly with the protonated Schiff base, leading to the blue-shift of the absorption spectrum. Our results with the Cl<sup>-</sup>-deficient state of gecko P521 do not seem to correlate with such a model of direct collapse. Although the absorption spectrum undergoes a blue-shift of 24 nm upon Cl<sup>-</sup> removal, we found that the Schiff base  $pK_a$  changes only slightly under these conditions (Fig. 9). This is inconsistent with the suggestion of a drastic change of charges in the immediate vicinity of the Schiff base, because such a change would most likely be accompanied by a significant change in the Schiff base  $pK_a$ . In fact, Wang et al. (1993) have proposed recently that the Cl<sup>-</sup>-binding site consists of H197 and K200 in human red and green pigments: these pigments have similar Cl<sup>-</sup> effects as gecko P521 (Wang et al., 1993). This binding site is located on the lumen surface of the gecko pigment (Kojima et al., 1992). This would suggest that the surface binding of the Cl<sup>-</sup> anion can induce in the interior of the pigment a further separation between the protonated Schiff base and its counterion, red-shifting the absorption maximum. But this change does not necessarily affect the  $pK_a$ , because the latter is probably associated with the orientation of the Schiff base and counterion groups and the intravening waters (Gat and Sheves, 1993). These properties are not necessarily affected much by the distance between the two groups. Further studies should be carried out to see whether, indeed, the Cl<sup>-</sup> binding affects the absorption maximum through perturbation in the Schiff base vicinity or other parts of the chromophore.

This work was supported in part by PHS EY01323 to TGE, the US-Israel Binational Science Foundation, and Human Frontier for the Promotion of Science to MS.

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