Chloride Binding Regulates the Schiff Base p*K* in Gecko P521 Cone-Type Visual Pigment†

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ABSTRACT: The binding of chloride is known to shift the absorption spectrum of most long-wavelength-absorbing cone-type visual pigments roughly 30 nm to the red. We determined that the chloride binding constant for this color shift in the gecko P521 visual pigment is 0.4 mM at pH 6.0. We found an additional effect of chloride on the P521 pigment: the apparent pK_a of the Schiff base in P521 is greatly increased as the chloride concentration is increased. The apparent Schiff base pK_a shifts from 8.4 for the chloride-free form to >10.4 for the chloride-bound form. We show that this shift is due to chloride binding to the pigment, not to the screening of the membrane surface charges by chloride ions. We also found that at high pH, the absorption maximum of the chloride-free pigment shifts from 495 to 475 nm. We suggest that the chloride-dependent shift of the apparent Schiff base pK_a is due to the deprotonation of a residue in the chloride binding site with a pK_a of ca. 8.5, roughly that of the Schiff base in the absence of chloride. The deprotonation of this site results in the formation of the 475 nm pigment and a 100-fold decrease in the pigment's ability to bind chloride. Increasing the concentration of chloride results in the stabilization of the protonated state of this residue in the chloride binding site and thus increased chloride binding with an accompanying increase in the Schiff base p*K*.

The light-induced deprotonation of the Schiff base of the chromophore in vertebrate visual pigments is a key event in the activation of the phototransduction cascade (*1*). The mechanism for this process is not yet understood, but it must involve a decrease in the proton affinity (pK_a) of the Schiff base. This leads to proton transfer to, and thus the neutralization of, the negatively charged counterion, glutamate 113, breaking the salt-bridge between these two charged groups. These events have been proposed to be a required part of the mechanism for transforming a visual pigment into its active conformation (reviewed in *2*, *3*). Thus, it is of great interest to elucidate the factors that affect and control the pK_a of the Schiff base. However, the high pK_a of bovine rhodopsin's Schiff base (>14, ref *4*) hinders identification of groups in rhodopsin which might control it, since in pH titration experiments alkaline denaturation of the pigment occurs before any possible alteration of the Schiff base pK_a by alteration of the opsin can be observed. Cone pigments are an appealing alternative system to study the control of the Schiff base pK_a. We have shown that the pK_a of the Schiff base of the cone-type visual pigment of gecko, P521, is much lower than that of bovine rhodopsin; in its native membrane environment, the Schiff base pK_a is 9.9 in 50 mM KCl (*5*).

In the present study, we show that the Schiff base pK_a of the gecko P521 pigment can be modulated by chloride binding. Previous studies of chloride binding to long-wavelength-absorbing cone pigments such as iodopsin, gecko P521, and the human green cone pigments have focused on chloride's effect on the pigment's absorption spectrum (*6*−*10*). It is well-known that chloride binding induces significant red-shifts of the absorption maxima of most long-wavelength cone pigments (26, 45, and 15 nm for gecko P521, chicken iodopsin, and the human green cone pigment, respectively) (*6*, *7*, *10*). Chloride binding, together with specific amino acid residues in the chromophore binding pocket (*11*−*13*), contributes to the spectral tuning of long-wavelength absorbing cone pigments. A chloride-induced spectral shift to the red seems to be present in most of the long-wavelength-absorbing cone pigments, with the exception of the mouse green cone pigment (*14*). The color-regulating chloride binding site in the long-wavelength cone pigments has been shown to be comprised of histidine 197 and lysine 200 (corresponding to glutamate 181 and glutamine 184 in bovine rhodopsin), located in the second extracellular loop of the pigment (*10*).

Our pH titration experiments with the gecko P521 pigment show that the apparent Schiff base pK_a increases by at least 2 pH units when the KCl concentration increases from 0 mM to 3 M. We find that this pK_a shift is independent of

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ionic strength or surface charge density and so is mediated through chloride binding. The dissociation constant for chloride implied by the Schiff base pK_a shift is at least 30 mM, 2 orders of magnitude higher than the dissociation constant for the binding implied by the spectral red-shift, 0.4 mM. Based on our results, we considered two models to explain the pK_a shift caused by chloride binding: The most likely one is that when the pH is raised to measure the pK_a of the Schiff base, the affinity of the pigment for chloride is reduced due to a deprotonation within the chloride binding site. The pigment in which the chloride binding site is deprotonated exhibits a blue-shift of its absorption maximum from 495 to 475 nm. An increase in chloride concentration would shift the equilibrium toward the protonated state of the binding site. This would keep the spectrum from blue-shifting due to chloride loss and would require that the chloride binding to the site found by Wang et al. (*10*) modulates the pK_a of the Schiff base. A less likely possibility is that there are two distinct binding sites for chloride. Binding of chloride to the high-affinity site leads to the red spectral shift while an additional binding to the low-affinity binding site ($K_d \geq 30$ mM) presumably results in an increase in the pK_a of the Schiff base. Both models reveal some intriguing aspects of the Schiff base pK and spectral tuning mechanisms.

MATERIALS AND METHODS

Preparation of Gecko Photoreceptor Outer Segments. Tokay geckos (West Coast Reptile, Fullerton, CA) were dark-adapted for at least 12 h at room temperature. The animals were then cooled to slow their movements, decapitated, and double-pithed under dim red light. The retinas were removed from the hemisected eyeballs and placed into buffer A (67 mM sodium phosphate, pH 7.0, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) on ice immediately. After homogenization of the retina with an 18 gauge needle, the membranes were subjected to a two-step sucrose flotation: first in buffer A plus 45% sucrose, then in buffer A plus 37% sucrose. The purified photoreceptor outer segments were stored in buffer A at −70 °C until needed.

Spectral Titration of Gecko P521 Visual Pigment. Absorption spectra of gecko P521 cone-type visual pigment, either in membranes or solubilized in 0.2% dodecyl maltoside (DM)[1] or 2% digitonin, were recorded with an Aviv 14DS spectrophotometer (Cary-Aviv Associates, Lakewood, NJ) at room temperature. The pK of the Schiff base we obtained from P521 titrated in DM is about 0.7 pH unit higher than the pK in digitonin. This may be due to the detergent's effect on the conformation of the pigment. A similar detergent-dependent pK_a shift has been seen for the Meta I−Meta II equilibrium in bovine rhodopsin (*15*). All pH titration data were fitted using KaleidaGraph (Synergy Software, Reading, PA) according to the Henderson−Hasselbalch equation to determine the pK_a and number of protons involved in the titration. The absorption maximum was determined by analyzing the spectral trace with a Gaussian function combined with fourth-order polynomials.

[1] Abbreviation: DM, dodecyl maltoside.

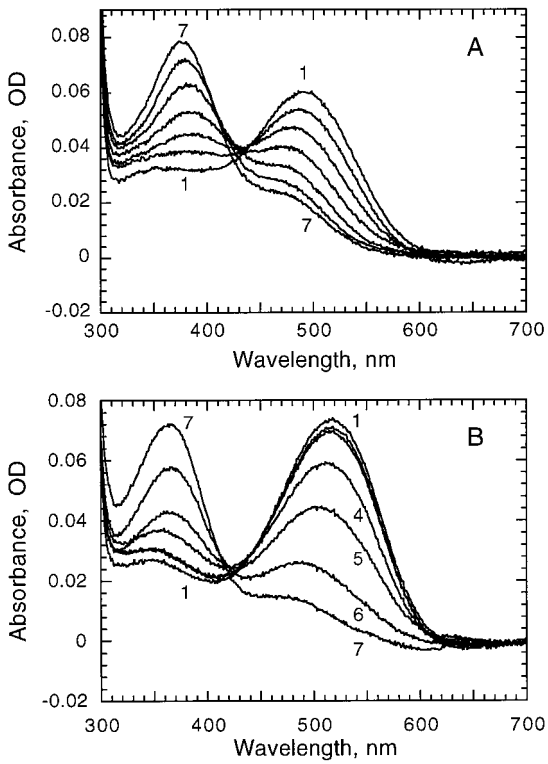

FIGURE 1: Absorption spectra of P521 cone-type visual pigment solubilized in 0.2% DM. (A) 0 mM KCl at pH: 6.7, 7.5, 8.1, 8.5, 9.0, 9.5, and 10.1, trace 1 through 7, respectively. (B) 0.2% DM, 50 mM KCl at pH: 7.2, 8.2, 8.6, 9.3, 9.6, 10.1, and 10.5, trace 1 through 7, respectively.

RESULTS

The Apparent pK_a of the Schiff Base Shifts with Increasing Concentration of KCl. The transformation of the gecko P521 pigment into a 380 nm species at high pH has been reported (*16*) and was shown to be due to the deprotonation of the Schiff base (*5*). Figure 1 shows the spectral changes in gecko P521 solubilized in 0.2% DM in 0 mM (Figure 1A) and 50 mM (Figure 1B) chloride with increasing pH. As the pH of the solution changed from neutral to alkaline, the absorption at 495 nm (chloride-free form of pigment; Figure 1A) or 521 nm (chloride-bound form; Figure 1B), which reflects the amount of the pigment with a protonated Schiff base, decreased, and the absorption near 380 nm, reflecting the amount of the deprotonated form of the pigment, increased. Figure 2 shows the pH titration curve of gecko P521 in its native membrane (Figure 2A). The apparent pK_a of the Schiff base in native membranes changed from 9.8 in 50 mM KCl to 11 in 4 M KCl.

Chloride Binding Raises the Apparent pK_a of the Schiff Base. The shift of the Schiff base pK_a to higher values with increasing [KCl] (Figure 2A) had been provisionally interpreted as being due to the salt-induced change in the surface potential associated with a positively charged membrane for gecko P521 (Gouy−Chapman effect; Liang & Ebrey, 38th Annual Meeting of Biophysical Society, 1994). However, the detergent-solubilized pigment (Figure 2B) shows the same pK_a increase with increasing [KCl] as the native membrane (Figure 2A), suggesting that, if it operates, this positive charge effect is due to the charges of the pigment itself rather than due to any other component of the membrane. While this is conceivably possible, further

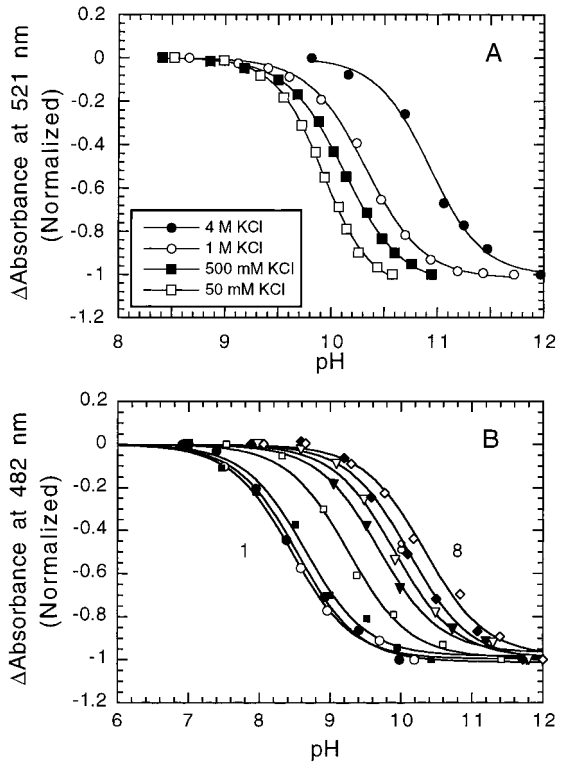

FIGURE 2: (A) pH titration of the gecko P521 cone-type visual pigment in photoreceptor outer segment membranes at various concentrations of KCl. (B) pH titration with the pigment solubilized in 2% digitonin, at constant ionic strength with varying [KCl]. Potassium acetate was used to adjust the ionic strength to 3 M. The concentration of [Cl^-] is 0 mM, 1 mM, 10 mM, 50 mM, 500 mM, 1 M, 2 M, and 3 M for curves 1 through 8, respectively.

probing of the surface environment by attachment of a pH-sensitive dye, 5-iodoacetate fluorescein, to cysteines of the pigment indicated a negatively charged environment for the probe (unpublished observations). Furthermore, we observed that alteration of the surface charge on the pigment by chemical modification of carboxyls also failed to affect the titration behavior (not shown).

To test if the KCl effect was a specific one by chloride rather than just the shielding of a positively charged surface by anions, we titrated gecko P521 pigment in the presence of K_2SO_4 (Figure 3A). Sulfate has been shown to have no effect on the absorption spectrum of the gecko P521 and other pigments (*10, 16*). Titration of the pigment at different sulfate concentrations in the presence of constant chloride (10 mM, to saturate the chloride binding site for spectral tuning; see below) did not show the pK_a shift seen with increasing KCl concentration as in Figure 2. Other anions such as acetate or phosphate which are similar to sulfate in having no effect on the spectral red-shift (*16*) also do not influence the Schiff base pK_a (data not shown). Nitrate, in contrast to chloride, can blue-shift the absorption maximum and has been shown to compete specifically with chloride for the chloride binding site (*10*); the pK_a of the Schiff base of gecko P521 shifts from 8.8 in 5 mM KNO_3 to 9.8 in 500 mM KNO_3 (Figure 3B).

Furthermore, to show that the equilibrium between the deprotonated and protonated Schiff base forms of pigment could be modulated by changing the concentration of chloride, the P521 pigment in 2% digitonin was incubated in 50 mM K_2SO_4 at pH 8.5 (Figure 3C). Since the pK_a of

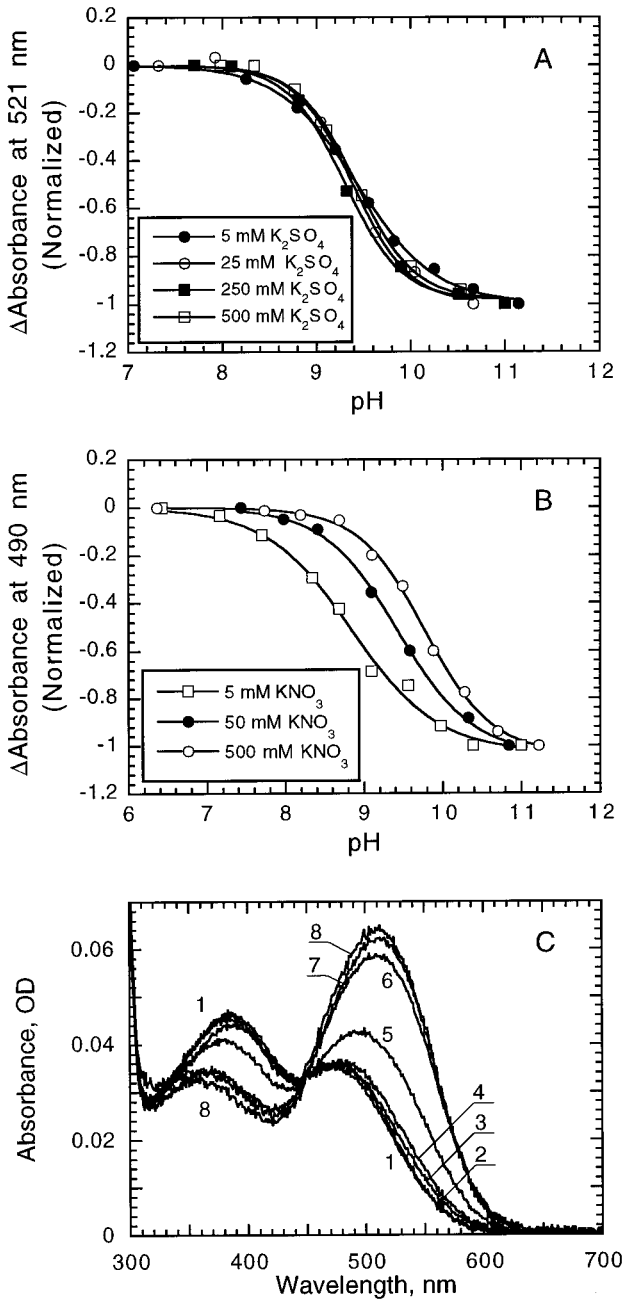

FIGURE 3: (A) pH titration of gecko P521 cone-type visual pigment in 0.2% DM with 10 mM KCl plus varying concentrations of K_2SO_4. The pK_a values are all 9.3−9.4. (B) pH titration with the pigment in 5, 50, and 500 mM KNO_3. The amplitude at 490 nm was used for the pK_a plot instead of 521 nm since nitrate blue-shifts the absorption maximum. The pK_a of the Schiff base shifts from 8.8 to 9.8 with increasing nitrate. (C) Chloride effect on the equilibrium between the protonated and deprotonated Schiff base forms of the pigment in 2% digitonin, 50 mM K_2SO_4, and 10 mM MOPS, pH 8.5. Chloride was added to the sample stepwise to make the final concentration 0, 0.1, 0.6, 1.1, 5.3, 55, 560, and 750 mM as represented by curves 1 through 8.

the Schiff base of P521 is 8.4 in the chloride-free state (Figure 2B, curve 1), about 50% of the pigment has a deprotonated Schiff base. This is reflected by the prominent absorption at 380 nm. As the chloride concentration is increased, the absorption at 521 nm increases, showing that protonation of the Schiff base occurs. This experiment also shows that the pigment is not denatured under the conditions we were using since the long-wavelength band could be recovered by adding chloride.

To show in a different way that the apparent p*K* shift was due to the change in chloride concentration rather than the change of ionic strength, the pigment was titrated in 2% digitonin at constant ionic strength but various KCl concentrations (Figure 2B). We have found that the pigment solubilized in digitonin appears to be a little more stable than pigment solubilized in DM, allowing more extensive titration experiments to be done. The ionic strength of the solutions was kept at 3 M by adding potassium acetate, which, as noted above, does not affect the pK_a of the Schiff base. The decrease of absorption at 482 nm (the isosbestic point of chloride-bound and chloride-free forms of pigment) was used to calculate the pK_a so that changes between the chloride-free and chloride-bound species would not distort the Schiff base pK_a measurements. At the highest KCl concentration used, 3 M, the pK_a was 10.4. Higher ionic strengths led to pigment denaturation. Interestingly, the chloride-induced pK_a increase does not saturate even in 3 M KCl (Figure 2B), and thus the pK_a of the Schiff base for the chloride-bound pigment cannot be precisely determined (see below).

Blue-Shift of the Absorption of the P521 Pigment at High pH: Evidence for Deprotonation in the Chloride Binding Site. We have found that when the pH is raised toward alkaline values, not only does the Schiff base deprotonate to form a 380 nm species, but also a blue-shift of the absorption maximum from 521 nm (chloride-bound form of pigment) or 495 nm (chloride-free form of pigment) to 475 nm is also observed (Figure 1A,B). This blue-shift suggests that a group which interacts with the chromophore (regulating its color) is altered or deprotonated as the pH is raised. Figure 4A illustrates this pH-induced blue-shift by plotting the long-wavelength absorption maximum of the protonated Schiff base form of the pigment against pH at various chloride concentrations. Regardless of the chloride concentration, the absorption eventually blue-shifted to around 475 nm at high pH. As shown in this figure, chloride obviously has two effects: it red-shifts the absorption maximum, and it raises the pH at which the long-wavelength absorption maximum shifts to 475 nm.

Previous studies of chloride binding by long-wavelength-absorbing cone-type visual pigments have focused on spectral tuning. Our results indicate that chloride binding has other effects besides red-shifting the absorption maximum. To further analyze these phenomena, we compared the chloride effect on the absorption maximum, the Schiff base pK_a, and the pH-induced blue-shift. First we determined that the dissociation constant for the red-shift due to chloride is 0.4 mM at pH 6.0. Pigments solubilized in 2% digitonin have about the same K_d as pigments solubilized in 0.2% DM (data not shown). The K_d of 0.4 mM is close to the value obtained for the chicken red cone pigment, iodopsin (0.1 mM; ref *6*, *9*), and the human green cone pigment (0.6 mM; ref *10*). Dose−response data for the chloride-induced spectral shift were obtained by Crescitelli (*7*); however, the conditions he used (pH 8.8 in 2% digitonin) gave an incorrect K_d (2.2 mM) due to the chloride binding site and the Schiff base being partially deprotonated (see below).

The chloride dependence on the apparent pK_a of the Schiff base can be inferred from the titration data in Figure 2B. We assumed that binding of chloride causes a change in the Schiff base pK_a by a magnitude of ΔpK_a. This couples the two reactions. The ΔpK_a characterizes the "coupling strength".

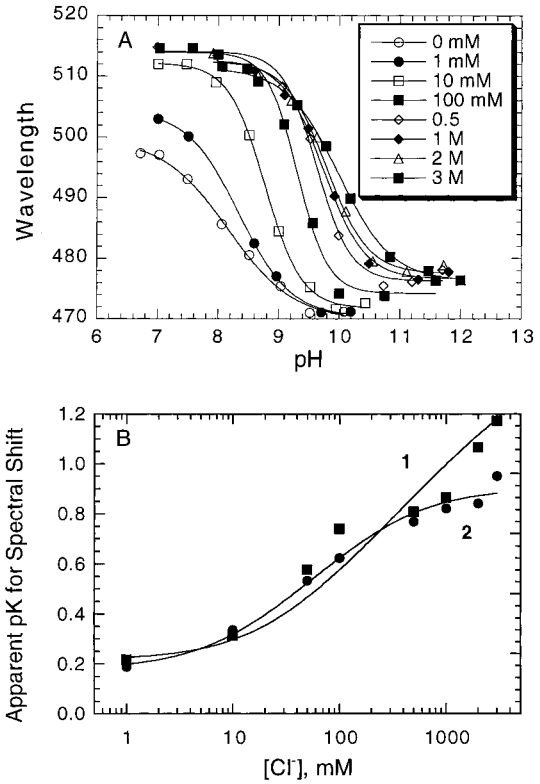

FIGURE 4: (A) pH dependence of the absorption maximum at various chloride concentrations. The transitions resemble titration curves and are curve-fitted with the Henderson−Hasselbalch equation to deduce a "pK_a". (B) Curve 1 (■), pK_a of the Schiff base at different chloride concentrations taken from the titration experiment shown in Figure 2B and fitted with eq 1; curve 2 (●), fitting of "pK_a" for the data from (A).

Considering equilibria in the coupled reactions of deprotonation of the Schiff base and chloride binding, it is easy to demonstrate that the apparent pK_a of the Schiff base depends on the concentration of chloride as described by eq 1:

$$pK_a([Cl^-]) = pK_1 + \log [(K_2 + [Cl^-])/(K_2 + [Cl^-]10^{-\Delta pK_a})] \quad (1)$$

where pK_a([Cl$^-$]) is the apparent pK_a of the Schiff base at a given chloride concentration, [Cl$^-$], pK_1 is the pK value of the Schiff base without chloride, K_2 is the dissociation constant of chloride, and ΔpK_a is the increase in the pK_a of the Schiff base upon chloride binding. The plot of the Schiff base pK_a's at different chloride concentrations is shown in Figure 4B (curve 1). The dissociation constant of chloride, K_2, deduced from the fit to the data by eq 1 is approximately 30 mM. However, since the shift of the Schiff base pK_a did not saturate at the highest [Cl$^-$] we used, the K_2 cannot be determined precisely. The ΔpK_a is at least 2.0.

The dependence of the absorption maximum upon pH (Figure 4A) was curve-fitted to the Henderson−Hasselbalch equation as the plot resembles a titration curve and the "pK_a" values were determined (curve 2, Figure 4B). If protonation of the chloride binding site is necessary for chloride binding and deprotonation of this site causes the absorption blue-shift at high pH (see Figure 5 for the proposed model), then the apparent binding constant of chloride to pigment should be pH-dependent. At high pHs, by mass action much higher chloride concentrations are needed in order to keep the

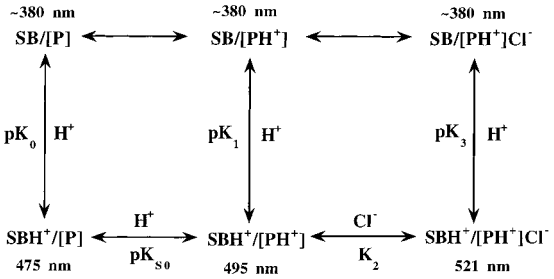

FIGURE 5: One chloride binding site model. Abbreviations: SB, deprotonated Schiff base; SBH^+, protonated Schiff base; P, pigment with the chloride binding site deprotonated; PH^+, pigment with the chloride binding site protonated; PH^+Cl^-, chloride-bound form of pigment; pK_0, the pK of the Schiff base when the chloride binding site is deprotonated; pK_{s0}, the pK of the chloride binding site; pK_1, the pK of the Schiff base when the chloride binding site is protonated while the pigment is chloride-unbound; K_2, the dissociation constant of chloride for the chloride binding site; pK_3, the pK of the Schiff base when the chloride binding site is protonated and the pigment is chloride-bound.

binding site in its protonated state. The apparent binding constant for the chloride site increases with increasing pH according to

$$K_2([H^+]) = K_2(1 + 10^{pH-pK_s}) \quad (2)$$

where $K_2([H^+])$ is the apparent dissociation constant of chloride at a specific pH, K_2 is the chloride dissociation constant at low pH, and pK_s is the apparent pK of the chloride binding site. The apparent pK_s of the binding site is also chloride-dependent. It increases with increasing chloride concentration according to

$$pK_s([Cl^-]) = pK_{s0} + \log(1 + [Cl^-]/K_2) \quad (3)$$

where pK_s([Cl^-]) is the apparent pK of the chloride binding site at a specific chloride concentration and pK_{s0} is the pK_a of the binding site in the absence of chloride.

Interestingly, curves 1 and 2 of Figure 4B have approximately the same pK_a value at each chloride concentration except perhaps at the highest concentrations. This suggests that these processes are closely related. If this is so, then the deprotonation of the group which is part of the chloride binding site causes the shift of absorbance to 475 nm and the decrease in the apparent pK_a of the Schiff base (see Discussion).

DISCUSSION

Chloride Binding Causes the KCl-Dependent pK_a Shift of the Schiff Base. In the present study we showed that the binding of chloride to the gecko P521 cone-type pigment can affect other properties of the pigment besides the absorption maximum. Chloride shifted the pK_a of the Schiff base of the pigment in 2% digitonin from 8.4 in 0 mM KCl to 10.4 in 3 M KCl (Figure 2B). This shift is not a surface charge effect because it is not influenced by chemical modification of the charged surface residues of the pigment (data not shown) or the ionic strength. Figure 3A shows that the ionic strength can be increased 100-fold without causing a pK_a shift. Moreover, if the ionic strength is kept constant, the pK_a increases as the concentration of chloride increases (Figure 2B). Thus, the pK_a increase upon increasing the chloride concentration is mediated through chloride binding to a special site on the pigment, not through a change in surface potential.

Evidence for Deprotonation of the Chloride Binding Site at High pH. The shift of the absorption maximum of the pigment in the chloride-free solution from 495 to 475 nm at high pH (Figures 1 and 4A) has not been described previously. The characteristic titration-like pH-dependence of the shift indicates that it is due to the deprotonation of a residue with pK_a ~8.2 in the absence of chloride. It is difficult to spectroscopically separate the 475 and 495 nm species because they form with similar pK_as and in parallel with the deprotonation of the Schiff base. One possibility is that the pH-induced blue-shift (521/495 nm → 475 nm) and deprotonation of the Schiff base (521/495 nm → 380 nm) are unrelated. However, they both show a similar dependence on chloride (Figure 4B) which leads us to suggest the following interpretation (see Figure 5). The chloride binding site (either the histidine or the lysine or both, ref *10*) should deprotonate at high pH. Pigment with a deprotonated chloride binding site absorbs at 475 nm. It does not bind chloride (or its affinity is hundred times lower). The deprotonation of the chloride binding site and/or chloride dissociation results in a decrease of the pK_a of the Schiff base by at least 2 pH units compared to its value in the functionally active P521 state.

One or Two Chloride Binding Sites? Modeling of the chloride titration data shows that in order to produce the chloride-dependent shift of the pK_a of the Schiff base we observed, K_2, the dissociation constant of chloride, must be about 30 mM (fitting of curve 1 in Figure 4B). This value is 2 orders of magnitude higher than the dissociation constant for the spectral shift induced by chloride (0.4 mM). Thus, one possibility is that there are *two distinct chloride binding sites* present on the gecko P521 pigment. The "high"-affinity site has a dissociation constant around 0.4 mM; when occupied by chloride, the absorption spectrum is red-shifted to 521 nm. The second binding site has a much lower affinity for chloride (30 mM). When occupied, the spectrum is not changed, but the Schiff base pK_a is significantly raised. Saturation of the high-affinity site with 5−10 mM chloride produces a 26 nm red-shift for the gecko P521 pigment but does not alter the Schiff base pK_a very much, as shown in Figure 2B.

A second, simpler possibility is that there is *one chloride binding site* whose affinity for chloride is pH-dependent as shown in Figure 5. In this model, binding of chloride [to the chloride binding site identified by Wang et al. (*10*)] causes a red-shift of the absorption spectrum as well as a significant pK_a shift of the Schiff base in gecko P521 cone-type pigment. Since the putative chloride binding site is comprised of one or possibly two positively charged amino acid residues, histidine 197 and lysine 200, neutralization of either one would result in partial or total loss of chloride binding capacity as demonstrated in mutagenesis experiments (*10*). It is very likely that at high pH, one or both of these residues become deprotonated and therefore the site can no longer bind chloride. Once chloride and a proton dissociate from the binding site, the pK_a of the Schiff base decreases and the Schiff base becomes deprotonated.

As shown in Figure 5, the *one binding site model* contains six pigment states which are in equilibrium with each other in accordance with the pH and chloride concentrations. In

chloride-free solution and at neutral pH, the pigment has a protonated Schiff base and a protonated chloride binding site (denoted as $SBH^+/[PH^+]$, $\lambda_{max} = 495$ nm). Raising the pH results in the deprotonation of the Schiff base (formation of SB/[P] plus $SB/[PH^+]$) as well as the chloride binding site ($SBH^+/[P]$, $\lambda_{max} = 475$ nm). The observation of the blue-shift from 495 to 475 nm accompanying the decrease of the protonated Schiff base form suggests that pK_1 (the Schiff base pK_a of the chloride-free pigment) is close to pK_{S0} (the apparent pK_a of the chloride binding site). If pK_1 were much lower than pK_{S0}, a decrease in absorption near 495 nm would precede the blue-shift as the pH is raised toward alkaline; if pK_1 were much greater than pK_{S0}, the blue-shift would be the predominant event during the pH titration process.

Addition of chloride would cause the spectral shift to the red and push the equilibrium toward the chloride-bound form of pigment ($SBH^+/[PH^+]\cdot Cl^-$, $\lambda_{max} = 521$ nm). When the chloride-bound form of pigment is titrated by increasing pH, the chloride binding site deprotonates and therefore the chloride dissociates. The Schiff base pK_a of the chloride-bound form (pK_3) must be greater than pK_{S0} (and thus greater than pK_1). Although the apparent pK_a of the chloride binding site (pK_s, which can be calculated using eq 3) increases as the chloride concentration increases, the deprotonation of the Schiff base always accompanies the blue-shift. This suggests that the deprotonation of the chloride binding site is required for the deprotonation of the Schiff base. Otherwise, the deprotonation process $SBH^+/[PH^+]\cdot Cl^- \rightarrow SB/[PH^+]\cdot Cl^-$ would occur and a decrease of absorption near 521 nm would be observed alone without any other evidence of the blue-shift to 475 nm.

Further evidence to support the single binding site hypothesis is from the data shown in Figure 3C. The gecko P521 pigment at pH 8.5 has an absorption maximum around 475 nm (at low pH, the absorption maximum for the chloride-free form of pigment is 495 nm). If this blue-shift were due to the deprotonation of some group(s) other than the chloride binding site, and the deprotonation affected the absorption of the pigment in a different manner than chloride binding, increasing the chloride concentration while keeping the pH constant at 8.5 would only cause a decrease at 475 nm. The fact that the addition of chloride not only shifts the equilibrium between deprotonated and protonated Schiff base forms of pigment but also shifts the absorption spectrum from 475 to 521 nm at high pH suggests that the deprotonation of the Schiff base and the blue-shift of the pigment to 475 nm at high pH both result from the deprotonation of the chloride binding site and the subsequent dissociation of the chloride. Interestingly, in human green cone pigment mutants, with the chloride binding site residues histidine and/or lysine neutralized by mutation, the absorption maximum is blue-shifted to 500 nm, compared to the chloride-bound form, 530 nm, and the chloride-free form, 515 nm, of the wild-type pigment (*10*). These would correspond to the 475, 521, and 495 nm states, respectively, of the gecko pigment.

The binding site deprotonation hypothesis can explain why the binding affinity of chloride is reduced by 2 orders of magnitude in the pK_a measurements (from 0.4 to 30 mM). Increasing the chloride concentration can prevent the deprotonation of the binding site from taking place by simple mass action that favors the protonated state of the chloride binding site. Therefore, the apparent pK of the Schiff base seems to increase with addition of chloride. The one-binding site model also explains why those anions (such as chloride and nitrate) that cause a spectral shift can act to increase the apparent pK in pH titration experiments and those anions that do not cause spectral shift (such as sulfate and acetate) fail to shift the Schiff base pK_a.

With either model for the effect of chloride on the pK, we can conclude that the pK of the Schiff base without chloride is ca. 8.2 and that with chloride is much greater, at least 10.4. At physiological chloride concentrations, the Schiff base pK will be about 9.8.

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