Octopus photoreceptor membranes

Surface charge density and pK of the Schiff base of the pigments

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ABSTRACT The chromophore of octopus rhodopsin is 11-*cis* retinal, linked via a protonated Schiff base to the protein backbone. Its stable photoproduct, metarhodopsin, has all-*trans* retinal as its chromophore. The Schiff base of acid metarhodopsin (λ_{max} = 510 nm) is protonated, whereas that of alkaline metarhodopsin (λ_{max} = 376 nm) is unprotonated. Metarhodopsin in photoreceptor membranes was titrated and the apparent pK of the Schiff base was measured at different ionic strengths. From these salt-dependent pKs the surface charge density of the octopus photoreceptor membranes and the intrinsic Schiff base pK of metarhodopsin were obtained. The surface charge density is $\sigma = -1.6 \pm 0.1$ electronic charges per 1,000 Å^2. Comparison of the measured surface charge density with values from octopus rhodopsin model structures suggests that the measured value is for the extracellular surface and so the Schiff base in metarhodopsin is freely accessible to protons from the extracellular side of the membrane. The intrinsic Schiff base pK of metarhodopsin is 8.44 ± 0.12, whereas that of rhodopsin is found to be 10.65 ± 0.10 in 4.0 M KCl. These pK values are significantly higher than the pK value around 7.0 for a retinal Schiff base in a polar solvent; we suggest that a plausible mechanism to increase the pK of the retinal pigments is the *preorganization* of their chromophore-binding sites. The preorganized site stabilizes the protonated Schiff base with respect to the unprotonated one. The difference in the pK for the octopus rhodopsin compared with metarhodopsin is attributed to the relative freedom of the latter's chromophore-binding site to rearrange itself after deprotonation of the Schiff base.

INTRODUCTION

Rhodopsin, an integral membrane protein, is the light-sensitive pigment of the photoreceptor cells of higher organisms. Photon absorption by rhodopsin initiates a biochemical amplification cascade that results in an electrical signal, which is then processed through the secondary neurons of the visual system (Stryer, 1986; Koutalos and Ebrey, 1986; Pugh and Cobbs, 1986; Tsuda, 1987).

Rhodopsin consists of the apoprotein opsin, and of a light-sensitive chromophore, which for almost all rhodopsins is 11-*cis* retinal (vitamin A aldehyde). The chromophore is linked to the protein backbone via a protonated Schiff base bond. The photochemistry of vertebrate and invertebrate rhodopsins is markedly different: the initial photochemical event, the isomerization of the chromophore around the 11-12 double bond, is the same; after that, vertebrate rhodopsins eventually hydrolyze to opsin and retinal, whereas invertebrate ones reach a stable state, metarhodopsin, with all-*trans* retinal as the chromophore.

In octopus, rhodopsin absorbs maximally at 475 nm. Metarhodopsin, the stable photoproduct, has two forms that are in a pH-dependent equilibrium: acid metarhodopsin, which has a protonated Schiff base and absorbs maximally at 510 nm, and alkaline metarhodopsin, which has a deprotonated Schiff base and absorbs maximally at 376 nm (Kitagawa and Tsuda, 1980; Tsuda et al., 1982; Pande et al., 1987).

Thus, the absorption spectrum of metarhodopsin is pH-dependent and can be used for monitoring the local pH, i.e., the proton concentration near the photoreceptor membrane's surface. From the Boltzmann equation, the local pH depends on the bulk pH and on the electrostatic surface potential of the membrane. From the Gouy-Chapman equation (McLaughlin, 1989) this surface potential is a function of just the membrane surface charge density and the ionic strength. Therefore, the pK of metarhodopsin, as measured by the bulk pH, would be expected to change with the ionic strength and in accordance with the Gouy-Chapman and Boltzmann equations.

In this paper, we have determined the surface charge density and the pK of metarhodopsin's Schiff base by monitoring the absorption spectrum of metarhodopsin vs. bulk pH at different ionic strengths. The surface charge density is an important quantity in understanding the electrostatics of protein-membrane interactions. Moreover, in combination with the pK of metarhodopsin it gives the ratio of acid/alkaline metarhodopsin under physiological conditions. This is of particular importance because metarhodopsin is important in the light-excitation pathway of the photoreceptor and, furthermore,

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photoconverts back to rhodopsin. The Schiff base pK is a direct measure of the affinity of metarhodopsin's chromophore-binding site for a proton. For comparison, we have also measured the rhodopsin's Schiff base pK. A major determinant of the Schiff base pK is the counterion, which is also responsible for the regulation of the pigment's spectrum (Honig et al., 1979; Koutalos et al., 1989*b*). So, the measured pK values are significant for refining and developing the models for spectral regulation in rhodopsin.

A preliminary account of this work has been presented at the Annual Meeting of the Biophysical Society (Koutalos et al., 1989*a*).

MATERIALS AND METHODS

Membrane preparation

Frozen octopus (*Paroctopus defleini*) eyeballs, dissected from octupi collected near Hokkaido, Japan, were shipped to Urbana for futher processing. The photoreceptor microvillar membranes were prepared according to Tsuda et al. (1986), and the lighter membrane fraction, rich in rhodopsin, was used for the titrations. The membranes were washed two more times with a solution of the relevant salt (KCl, NaCl, or $MgSO_4$), with no buffer added, and finally suspended in a solution of known salt concentration. The final salt concentration was adjusted before measuring by additions of concentrated salt solutions (4 M KCl or NaCl, and 2 M $MgSO_4$). The concentrations of the stock solutions used were calculated from the directly measured volumes and weights.

Spectral measurements

Spectra were obtained with an Aviv 14DS spectrophotometer with an end on photomultiplier. A membrane sample of 2 ml in a quartz cuvette was used for each titration. The membranes were magnetically stirred throughout the experiment, and so the photomultiplier was positioned farther away from the cuvette to minimize the magnetic effect from the stirrer. A baseline spectrum of the same cuvette, with 2 ml of distilled water magnetically stirred, was subtracted from all spectra.

Metarhodopsin-containing membranes were produced by irradiating the sample in the cuvette with blue light (420-nm interference filter) from a 300-W projector, for 5 min. As estimated from the spectra, there was <20% of rhodopsin left unconverted after this, but it is not expected to interfere with the metarhodopsin titration because of rhodopsin's much higher pK (pK difference of >2 pH units).

For pH adjustment, solutions of varying normalities (1.0 N, 0.1 N, and 10 mN) of KOH and HCl were used in the case of KCl and $MgSO_4$ salts; instead of KOH, NaOH solutions were used with NaCl. Acid or base were added in volumes not exceeding 20 μl per addition, and each spectrum was corrected for dilution. The pH was recorded with an open glass electrode (Beckman Instruments, Inc., Fullerton, CA or Radiometer America Inc., Westlake, OH). Usually (and depending on the ionic strength) a few minutes were enough for equilibration, as judged by the stabilization of the pH meter's reading, and then the pH value was recorded.

All the sample manipulations and measurements were carried out under dim red light at a room temperature of 23°C. Doubly distilled water was used and all reagents were of analytic grade.

Data processing

The pH-induced absorption change was measured from difference spectra as follows: A reference spectrum was recorded at a sufficiently low pH (the pH of the unbuffered solution in which the membranes were suspended), where practically all of the pigment could safely be assumed to be in the acid form. This spectrum was subtracted from each of the other spectra recorded at higher pHs, and the titrated pigment was measured as the absorption loss at 510 nm (for metarhodopsin) or 475 nm (for rhodopsin). Measuring this absorption loss was not straightforward because pH-dependent light-scattering changes accompanied the absorption changes. The scattering contribution was corrected for by subtracting from the difference spectra a spectrum for the scattering background. The presence of the isosbestic points and the lack of any scattering beyond 650 nm in the corrected spectra corroborated the effectiveness of the procedure.

To obtain the apparent pK at each ionic strength, the absorption change δA (measured as absorption loss) was plotted vs. the bulk pH (measured by the electrode). A three-parameter curve was subsequently fitted to the points and the pK was obtained. The curve is given by:

$$\delta A = A_m/\{1 + 10^{[n\cdot(pK - pH)]}\},$$

where the three parameters are: A_m, the total amount of titratable pigment in units of absorption at 510 nm (or 475 nm); n, the number of protons involved in the transition; and pK, the midpoint of the titration. The equation was obtained by algebraic manipulation of the binding equation:

$$K^n = [H^+]^n \cdot [P^-]/[H_nP],$$

where P^- and H_nP the pigment's unprotonated and protonated forms, respectively.

Linearization of the Gouy-Chapman equation

To obtain the intrinsic pK of the Schiff base and the membrane surface charge density from the apparent pKs, the Boltzmann and the Gouy-Chapman equations were used:

Boltzmann:

$$[H^+]_{local} = [H^+]_{bulk} \cdot \exp(-e\phi_0/kT), \quad (1)$$

where local refers to the proton concentration on the membrane surface (the one affecting the pigment spectrum); bulk refers to the proton concentration in the bulk solution (the one measured by the electrode); e is the proton charge; ϕ_0 is the electrostatic potential at the membrane surface; k is Boltzmann's constant; and T is the temperature in degrees Kelvin.

Gouy-Chapman:

$$A \cdot \sigma/C^{1/2} = \sinh(ze\phi_0/2kT), \quad (2)$$

where σ is the membrane surface charge density; C is the salt concentration; z is the electrolyte's valence (the equation in this form applies to a symmetric electrolyte); and A is a constant that equals 136.4 $M^{1/2} \cdot Å^2$ at 22°C (McLaughlin, 1977). At large negative ϕ_0 Eq. 2 becomes

$$4 \cdot A^2 \cdot \sigma^2/C = \exp(-ze\phi_0/kT). \quad (2')$$

We define the "intrinsic" pK (pK_i) of the pigment's Schiff base as the

local pH at which the titration midpoint is reached. So, at a bulk pH equal to the apparent pK of the pigment, the local pH would be equal to the pK_i of the pigment. From this and Eq. 1,

$$pK_i - pK = \log[\exp(e\phi_0/kT)]. \quad (3)$$

By substituting Eq. 3 for $\exp(e\phi_0/kT)$ in Eq. 2, and after algebraic manipulations we obtain:

$$Y = S \cdot X + B \quad (4)$$

with $Y = 10^{z \cdot pK}$; $X = 10^{z \cdot pK/2} \cdot C^{-1/2}$; $pK_i = 1/z \cdot \log B$; $\sigma = -S/(2 \cdot A \cdot B^{1/2})$. Y and X can be calculated directly from quantities already measured and known (z, pK, and C). By plotting Y vs. X and fitting a least-squares straight line to the points, the two constants S and B are obtained and from those the intrinsic pK and the surface charge density are calculated.

RESULTS

Reversibility

Fig. 1 *a* shows difference spectra from the titration of metarhodopsin in 200 mM KCl. The major feature is the drop of absorption at 510 nm and the concomitant increase of absorption at 380 nm with increasing pH. Because free retinal absorbs maximally in the 380-nm region as well, the changes might be due to protein denaturation at high pH. Such denaturation is expected to be irreversible (see e.g., Radding and Wald, 1956); therefore, the reversibility of the absorption changes with decreasing pH would rule this possibility out and establish the origin as the acid/alkaline transition. This reversibility is shown in Fig. 1 *b* for metarhodopsin in 200 mM KCl. All recorded titrations were reversible within 15% of the total titrating pigment (as measured by A_m). This uncertainty could be due either to a partial denaturation of the pigment or to a heterogeneity of the membrane preparation with respect to proton permeability. With respect to the second possibility, the addition of permeabilizing, but subsolubilizing, concentrations of the detergent Triton X-100 showed the amount of nontitrating pigment to be within the upper limit of 15% mentioned above (data not shown).

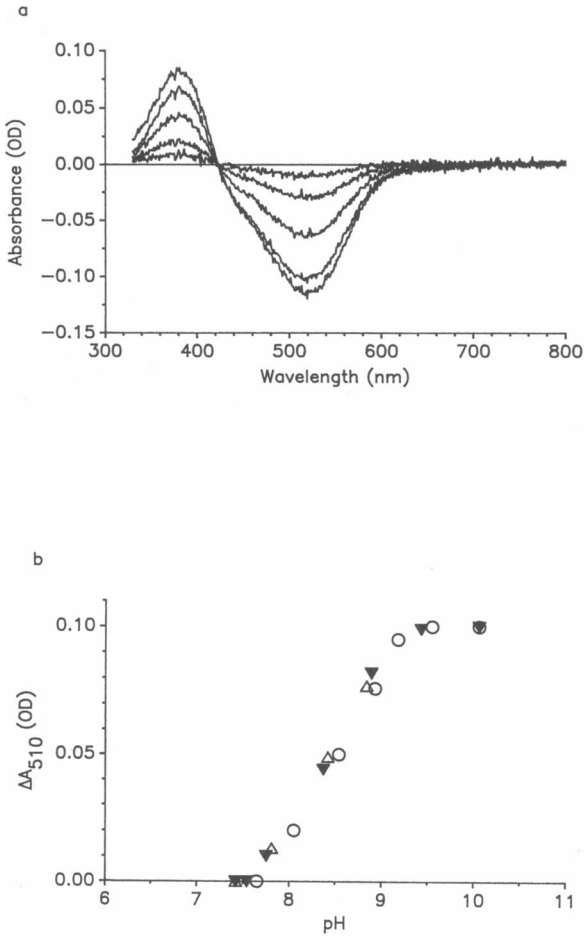

FIGURE 1 (*a*) Difference spectra from octopus metarhodopsin titration in 200 mM KCl. The reference spectrum is at pH 7.04 (zero line); the other pHs, in the order of decreasing absorbance at 510 nm, are 7.68, 8.17, 8.70, 9.37, and 9.81. (*b*) Reversibility of metarhodopsin titration in 200 mM KCl. The pH was increased (○), then decreased (▼), and finally increased again (△). The data for *a* and *b* are from different samples.

Salt-dependent pKs of metarhodopsin

Metarhodopsin-containing membranes were titrated at different KCl concentrations. Fig. 2 shows the dependence of metarhodopsin's pK on ionic strength as a shift in the titration curve with increasing KCl concentration. To demonstrate that the change is not ion-specific (i.e., due to specific interactions between K^+ or Cl^- and metarhodopsin), alternative salts were used as well. Fig. 3

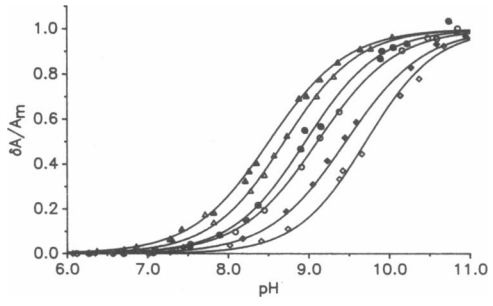

FIGURE 2 Shift of the metarhodopsin titration curve by increasing salt concentrations (KCl): 5 mM (◇), 23 mM (◆), 53 mM (○), 103 mM (●), 503 mM (△), 2.0 M (▲). The curves and data points have been normalized with respect to the total amount of titratable pigment.

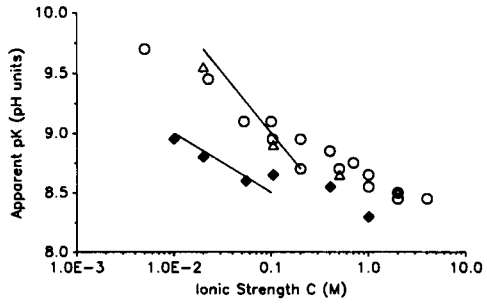

FIGURE 3 Plot of the apparent pK vs. the logarithm of ionic strength for octopus metarhodopsin: KCl (○), NaCl (△), and $MgSO_4$ (◆). Lines with slopes −1 and −0.5 have been drawn to guide the eye (see text for details).

compiles the recorded pKs at different salt concentrations and with alternative anions and cations: Na^+ has the same effect as K^+, whereas $MgSO_4$ is far more effective in screening the surface charges. By combining Eqs. 2′ and 3, it can readily be seen that at large negative surface potentials (which correspond to low ionic strengths) the slope of the pK vs. the log C graph should be −1 for monovalent and −0.5 for divalent electrolytes. This is approximately the case for Fig. 3, and is therefore consistent with the predictions of the Gouy-Chapman equation.

In the case of KCl and NaCl it was possible to obtain almost complete (>90%) titration curves. Unfortunately, this was not possible for $MgSO_4$, due to the limited solubility of $Mg(OH)_2$. In this case the titrations were 60–70% complete, resulting in significantly larger errors in the determination of the pK. They were nevertheless carried out to provide an additional control for the behavior of the surface potential according to the Gouy-Chapman equation.

For each curve-fitting of the titration data, the number n of protons involved in the acid to alkaline transition was obtained. The average, calculated by pooling together the numbers from all the metarhodopsin titrations performed, was $n = 0.94 \pm 0.02$. The minimum value of n was 0.90 and the maximum was 1.10. These numbers are consistent with the acid/alkaline transition involving a single proton, presumably the one at the Schiff base (see discussion).

Surface charge density and pK_i of metarhodopsin

The surface charge density σ and the pK_i of metarhodopsin can be obtained from the linearized Gouy-Chapman Eq. 4. This is shown in Fig. 4 *a* for KCl, for

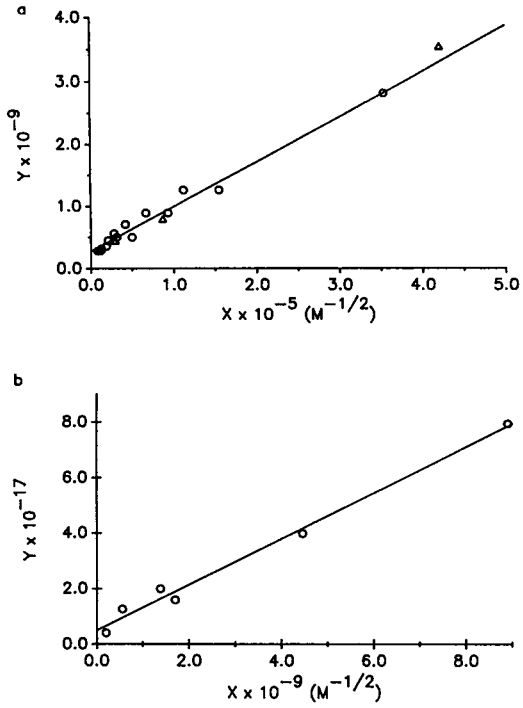

FIGURE 4 (*a*) Plot of $Y = 10^{pK}$ vs. $X = 10^{pK/2} \cdot C^{-1/2}$ for the monovalent salt-dependent metarhodopsin pKs; (○) KCl; (△) NaCl. (*b*) Plot of $Y = 10^{2pK}$ vs. $X = 10^{pK} \cdot C^{-1/2}$ for salt-dependent metarhodopsin pKs in $MgSO_4$. The lines are least-squares fits to the points (see text for details).

which $z = 1$. From the least-squares straight line, the intersection with the Y-axis and the slope were obtained:

$$B = (0.273 \pm 0.034) \times 10^9 \text{ and}$$

$$S = (0.721 \pm 0.030) \times 10^4 M^{1/2},$$

and from those, $pK_i = 8.44 \pm 0.12$ and $\sigma = -1.6 \pm 0.1$ electronic charges per 1,000 Å^2 were calculated.

Fig. 4 *b* shows the plot for $MgSO_4$, for which $z = 2$. Similarly, the intersection and the slope were obtained:

$$B = (0.490 \pm 0.184) \times 10^{17} \text{ and}$$

$$S = (0.826 \pm 0.044) \times 10^6 M^{1/2}$$

and from those, $pK_i = 8.35 \pm 0.19$ and $\sigma = -1.4 \pm 0.3$ electronic charges per 1,000 Å^2 were calculated.

The relatively small errors ($\delta B/B = 12\%$ and $\delta S/S = 4\%$) in the case of KCl justify the use of the Gouy-Chapman equation for relating the membrane surface potential to the ionic strength. Consistent with this is that the $MgSO_4$ values are in agreement with the ones obtained from KCl, though the errors are larger ($\delta B/B = 38\%$ and $\delta S/S = 5\%$), as expected.

Rhodopsin pK

It was not possible to obtain the pK of the deprotonation of rhodopsin's Schiff base in the same way as for metarhodopsin. The main reason is that this pK is significantly higher, e.g., at 200 mM KCl it is ~11.2 (data not shown) compared to 8.80 for metarhodopsin. At the very high pHs required for titration rhodopsin denatures, making titration possible only up to ~60% conversion. In addition, titration at that pH range would involve deprotonation of amino-acid side chains (tyrosines and lysines) that would change the surface charge density, making the Gouy-Chapman equation inapplicable. For these reasons rhodopsin's pK was measured in 4 M KCl. At this ionic strength the pK of metarhodopsin is 8.45, i.e., the same as the pK_i. Therefore, 4 M KCl was sufficient for eliminating the surface potential and the adverse effects of changing surface charge density, and comparing metarhodopsin and rhodopsin under these conditions is valid.

Fig. 5 *a* shows difference spectra from the rhodopsin titration in 4 M KCl. The alkaline form has an absorption maximum around 374 nm, and its isosbestic point with the acid form is around 418 nm, as measured from the difference spectra. The titration curves of rhodopsin and metarhodopsin in 4 M KCl are shown in Fig. 5 *b*, and the rhodopsin pK is 10.65 ± 0.10. Rhodopsin's titration was reversible (Fig. 5 *c*), as well as metarhodopsin's (data not shown for 4 M KCl).

Estimation of errors

The magnitude of the errors in the Y and X values for the plots of Fig. 4 is very important for assessing the suitability of the linearized Gouy-Chapman equation for calculating the intrinsic pK and the surface charge density. The error in measuring the pK at a particular salt concentration is dominated by the uncertainty in A_m, which is at most 15%. This gives an error $\delta pK = 0.1$ (the uncertainty in the pK from the actual fitting is <0.05). From this we get for the KCl plot $\delta Y/Y = 23\%$, and for the $MgSO_4$ plot $\delta Y/Y = 46\%$. For the X values the error in the measured ionic strength C contributes as well. For C there are three sources of error: in measuring salt weights, in measuring solution volumes, and in the changes in the ionic strength during the titration because of the addition of K^+. The errors in weight and volume are <1%; the total amount of added K^+ during each titration was 3–5 mM. This would introduce an error of >100% for $\delta X/X$ in the case of the 5 mM KCl and for this reason this point was not included in the plot. For ionic strengths >20 mM KCl the error $\delta C/C$ becomes <20% and δX is dominated by δpK. In the case of $MgSO_4$, δX is dominated by δpK at all concentrations because divalents are far more effective in screening than

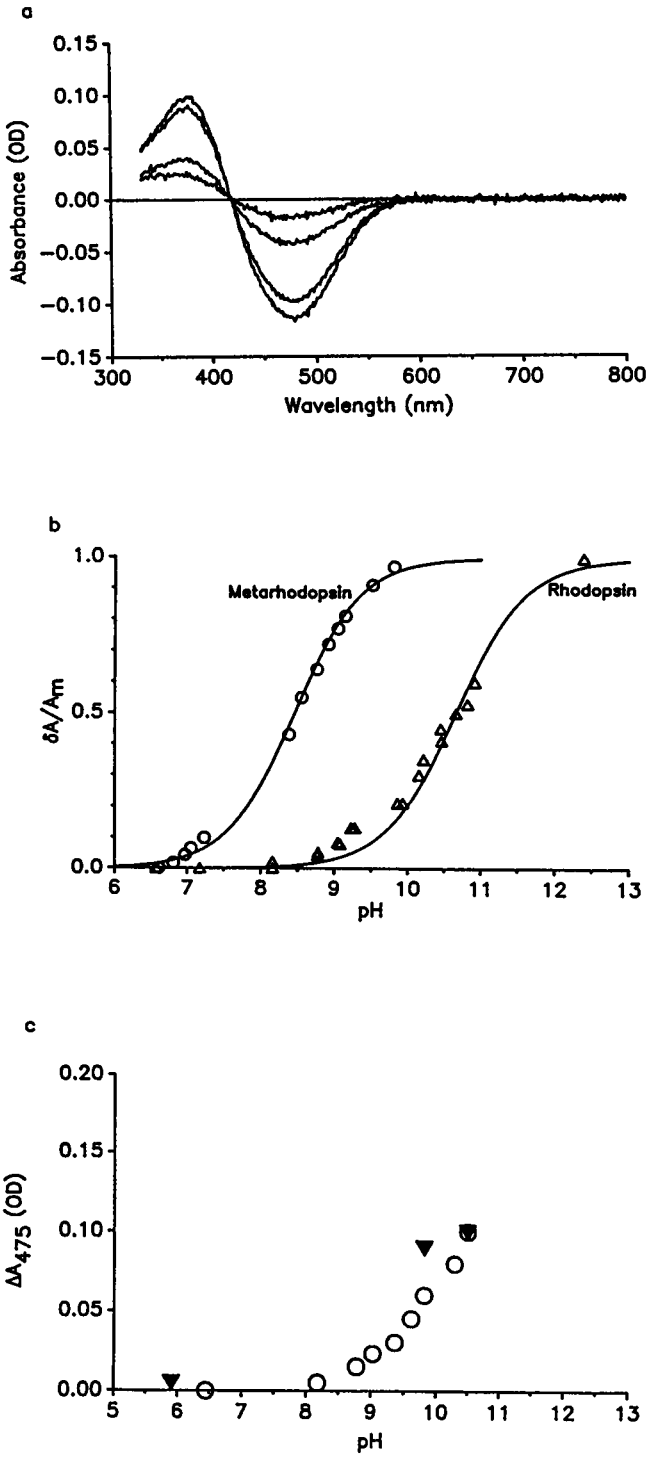

FIGURE 5 (*a*) Difference spectra from rhodopsin titration in 4 M KCl. The reference spectrum is at pH 6.57 (zero line); the other spectra, in the order of decreasing absorbance at 475 nm, are at pHs 9.05, 9.86, 10.66, and 10.92. (*b*) Titration curves of rhodopsin and metarhodopsin in 4 M KCl. Data points and curves have been normalized with respect to the total amount of titratable pigment. The rhodopsin titration endpoint was obtained by denaturing (raising the pH to 12.39). (*c*) Reversibility of rhodopsin titration in 4 M KCl. The pH was increased (○), and then decreased (▼). The total amount of rhodopsin was ~0.2 OD (measured at 475 nm).

monovalents; so, the added K^+ is not expected to affect the $MgSO_4$ ionic strength significantly. Therefore, $\delta X/X$ is 10–20% for KCl and 23% for $MgSO_4$.

Based on these values we relied on the plot from the KCl titrations to compute pK_i and σ, and used the $MgSO_4$ plot to corroborate the result.

DISCUSSION

The first question to be asked in view of the results of this study is the identity of the titrating group in rhodopsin and metarhodopsin. This group is responsible for the pigments' absorption changes upon raising the pH. There is strong evidence that in both cases these changes are due to the deprotonation of the Schiff base. In the case of metarhodopsin, resonance Raman experiments (Kitagawa and Tsuda, 1980) indicate that the large shift to shorter wavelengths upon raising the pH is due to the deprotonation of the Schiff base. For rhodopsin, the absorption change upon raising the pH is quite similar, and Schiff base deprotonation is the simplest way of explaining it.

The second step is the assignment of the pK of the absorption changes to the deprotonation of the Schiff base. This is the simplest model consistent with the observation that only one proton is released during the spectral transition from acid to alkaline metarhodopsin (also observed by Cooper et al., 1986). More complicated models would have to involve at least two additional protons (one taken up, one more released), for a total of three. For bovine rhodopsin, the metarhodopsin I to II transition is such a complicated process; in total, one proton is taken up, and the Schiff base gets deprotonated (Doukas et al., 1978; Parkes and Liebman, 1984).

The intrinsic pK that we measure (8.44 ± 0.12) for octopus metarhodopsin *in situ* is significantly different from the one Cooper et al. (1986) measured in the detergent L1690 (9.5), with 80 mM Tris or borate buffer; it is unclear whether this value would decrease much in very high salt. The difference may be reconciled by the report of Nashima et al. (1980) that detergents significantly shift the Schiff base pK of invertebrate rhodopsins and metarhodopsins.

The problem of pK

One important feature of the models that have been proposed for the protein-chromophore interaction in retinal-containing pigments is the negatively charged counterion which stabilizes the positive charge on the Schiff base (Honig et al., 1979; Koutalos et al., 1989*b*). The usual assumption about the counterion is that it is a carboxylate, which hydrogen bonds directly to the Schiff base. This carboxylate is the source of a negative electrostatic potential. Decreasing this potential should both red shift the spectrum and decrease the pK of the Schiff base. In the following we will discuss pigments for which sufficient information exists for the Schiff base environment. Apart from octopus rhodopsin and metarhodopsin, they include bacteriorhodopsin (a bacterial pigment) and to a limited extent bovine rhodopsin.

There is some evidence to suggest that the Schiff base environment is more complex than a single carboxylate group (Birge et al., 1988; Deng and Callender, 1987; Gilson and Honig, 1988). One proposal is that a carboxylate stabilizes the Schiff base without hydrogen bonding to it directly, and the nitrogen hydrogen bonds to a polar but neutral group. It may even be possible that a group of dipoles, or possibly one end of an α-helix (Honig, 1987), stabilize the positive charge on the Schiff base, with no carboxylate nearby. In the case of bovine rhodopsin, replacing glutamic acid 113 by glutamine changes the pK of the Schiff base dramatically (Zhukovsky and Oprian, 1989; Sakmar et al., 1989), making this carboxylate a candidate for the counterion. But, this pK change may also be due to a mutation-induced protein conformational change that involves the "opening up" of the chromophore-binding site to the external solution (see below). In addition, there is no corresponding amino acid to Glu-113 in octopus rhodopsin, whereas the dihydro-chromophore experiments have suggested that the octopus and bovine rhodopsin chromophore-binding sites are fairly similar (Koutalos et al., 1989*b*). In the case of bacteriorhodopsin, replacing aspartic acid 85 by alanine or asparagine, lowers the pK of the Schiff base to 8.2 and 7.0, respectively (Otto et al., 1990), making this carboxylate the counterion candidate.

Resonance Raman experiments on octopus rhodopsin and acid metarhodopsin (Kitagawa and Tsuda, 1980; Pande et al., 1987) show that the stretching frequency for the C=N bond for both pigments is at 1,655 cm^{-1}. This is the same frequency found in model compounds (in certain solvents and with certain counterions; Baasov et al., 1987), implying that the strength of the hydrogen bond of the Schiff base in the pigments and in the model compounds is the same.

For bacteriorhodopsin, a weakened Schiff base-counterion interaction has been proposed (de Groot et al., 1989). This would mean that the nitrogen-counterion bond length in bacteriorhodopsin is increased with respect to that of model compounds, thus red shifting the spectrum and decreasing the strength of the hydrogen bond. This conclusion is consistent with Raman (Deng and Callender, 1987; Gilson and Honig, 1988), NMR (Harbison et al., 1985) and absorption spectroscopic measurements (Lugtenburg et al., 1986; Spudich et al., 1986).

The pK of the Schiff base in bacteriorhodopsin is above 13.0, in octopus rhodopsin is 10.65, and in octopus metarhodopsin is 8.44. The pK of a Schiff base in model compounds is ~7.0 (Sheves et al., 1986). The pK of the Schiff base should be a monotonic function of the interaction with the counterion (the stronger the interaction, the higher the pK). More generally, the pK would be expected to correlate with the electrostatic potential, whatever the potential's source. This expectation conflicts with the experimental pKs, which definitely show no such correlation with the electrostatic potential, as indicated by the C=N frequencies.

A possible resolution of this difficulty can be obtained by recognizing that the pK of the Schiff base is determined by the difference in free energy between its protonated and unprotonated form. In polar solvents, the solvent molecules have the freedom to optimize their interaction with both the protonated and unprotonated Schiff bases simply by reorienting as a proton is taken up or released. If, on the other hand, the charged and polar groups in the protein are preorganized so as to optimize their interaction with the protonated Schiff base, they would not be as free to reorient when a protein is released. This would have the effect of stabilizing the protonated form relative to the unprotonated form and hence increasing the pK of the group.

Semi-empirical quantum mechanical calculations (Birge and Hubbard, 1980; Birge, 1990) show that in the protonated Schiff base the charge density on the nitrogen is ~ -0.21 au, while the charge density on the Schiff base proton is ~0.16. In the unprotonated Schiff base the charge density on the nitrogen becomes ~ -0.27. Thus, a counterion on the protein with a negative partial charge which stabilizes the Schiff base proton would find itself repelled by the partial negative charge on the nitrogen in the unprotonated Schiff base. This would tend to destabilize the unprotonated state and hence increase the pK of the Schiff base.

Extending the same reasoning to the octopus rhodopsin and metarhodopsin pigments, the measured pKs indicate that the unprotonated Schiff base interacts differently with its environment in the two species. The fact that the C=N stretching frequency is so similar in rhodopsin and metarhodopsin suggests that electrostatic interactions between the protonated Schiff base and the protein are also similar (Baasov et al., 1987) and thus would not be expected to contribute to the difference in pKs. If, on the other hand, the protein was freer to rearrange itself upon Schiff base deprotonation in the metarhodopsin state, this would allow greater stabilization of the unprotonated species and hence produce a lower pK. This explanation is consistent with the relative susceptibility of invertebrate metarhodopsin to hydroxylamine attack, while rhodopsin is stable in the presence of hydroxylamine (Hubbard and St. George, 1958). These observations suggest that the Schiff base region is more accessible to solvent in metarhodopsin and hence is more likely to be able to adjust its conformation upon Schiff base deprotonation.

Surface charge density

The model on which the Gouy-Chapman equation is based approximates the membrane surface with an infinite sheet of uniform charge density (McLaughlin, 1977). In the case of the octopus photoreceptor membranes, the charges are from the surface amino acids of rhodopsin and membrane lipids. These charges are discrete and comprise a nonuniform charge density at the local level. Nevertheless, the Gouy-Chapman approximation is the simplest one to use, albeit a crude one. Another crucial assumption is that the surface charge density remains approximately the same during each titration. This is required so that the relation of the local pH to the bulk pH does not change during the course of a single titration. Therefore, our calculations assume that the only group titrating is the Schiff base. Other groups that might titrate in the same region are the cysteines (intrinsic pKs around 9.0) and the amino groups in phosphatidylserine and phosphatidylethanolamine (intrinsic pKs in the range of 9.5–10). The reasonable fits to the data indicate that these groups do not present a problem.

Octopus rhodopsin has been sequenced, and a folding scheme has been proposed (Ovchinnikov et al., 1988*b*; Fig. 6). From this scheme we have calculated that in the 7.4–9.4 pH region there are -4 extracellular and 0 intracellular charges per rhodopsin. In addition to the amino-acid charges, there are 2–3 negatively charged lipids per rhodopsin (Akino and Tsuda, 1979; Paulsen et al., 1983). Assuming that these lipids are equidistributed among the two membrane bilayer leaflets we arrive at -5.5 extracellular and -1.5 intracellular charges per rhodopsin. The assumption of the equidistribution of the charged lipids does not hold in the case of bovine rhodopsin, where lipid distribution is asymmetric, driven by the charge asymmetry on rhodopsin (Hubbell, 1990). In the case of octopus the rhodopsin is not as asymmetric as for bovine, and the number of charged lipids is far less; therefore we do not expect our conclusions to qualitatively change by assuming the equidistribution. By further assuming a rhodopsin membrane density of 27,000–30,000 molecules/μm^2 (similar to bovine's), we obtain a surface area of ~3,500 $Å^2$ per rhodopsin. This gives -1.6 charges per 1,000 $Å^2$ for the extracellular side and -0.4 charges per 1,000 $Å^2$ for the intracellular side. These numbers, in conjunction with the experimental value for the surface charge density of -1.6 ± 0.1 charges per 1,000 $Å^2$, suggest that it is from the extracellular side that we are titrating metarhodopsin's Schiff base. This is

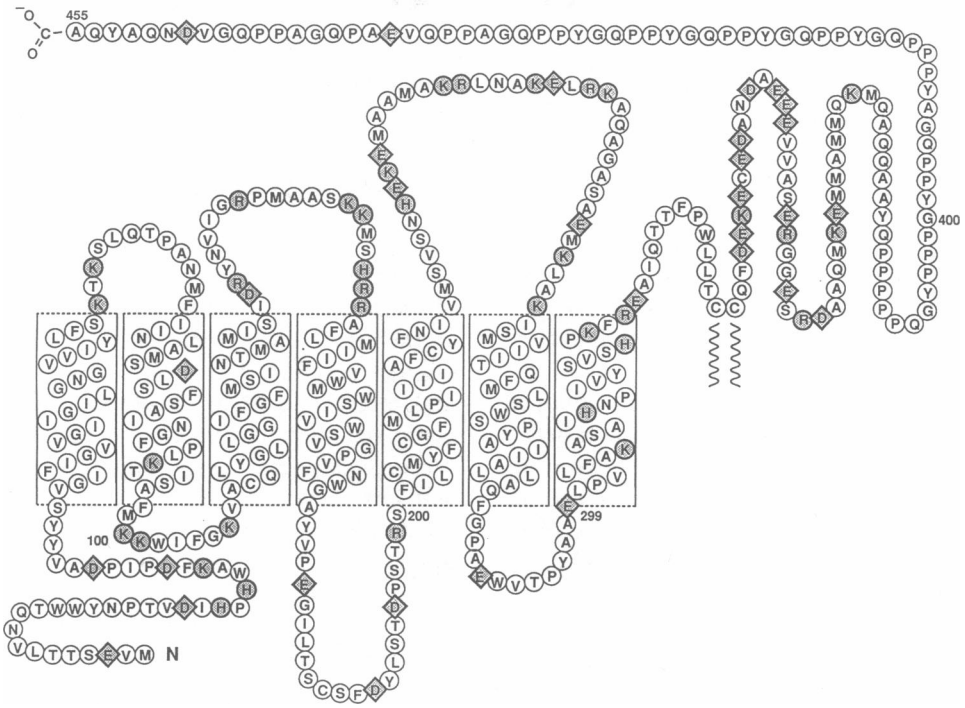

FIGURE 6 Octopus rhodopsin sequence and transmembrane folding (adapted from Ovchinnikov et al., 1988*b*). We have added fatty acid chains to the cysteines 337 and 338, providing an additional intracellular loop, based on a similar finding for bovine rhodopsin (Ovchinnikov et al., 1988*a*).

consistent with the experiments of Lisman et al. (1981), carried out with electrophysiological techniques. They concluded that in *Limulus* the pH-sensitive site for the acid-alkaline metarhodopsin transition is on the extracellular surface of the plasma membrane.

Under physiological conditions, i.e., in seawater (see Prosser and Kirschner [1973] for interstitial fluid and seawater compositions) the apparent pK of metarhodopsin could be as low as 8.6–8.7, corresponding to a surface potential of ~ -12 mV. Because the extracellular fluid pH can be as high as 8.1, this means that in vivo as much as 20% of metarhodopsin may be in the alkaline form. Because the enzymatic properties of alkaline metarhodopsin are currently unknown, its physiological relevance remains unclear.

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