pK_a of the protonated Schiff base of bovine rhodopsin
A study with artificial pigments

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ABSTRACT Artificial bovine rhodopsin pigments derived from synthetic retinal analogues carrying electron-withdrawing substituents (fluorine and chlorine) were prepared. The effects of the electron withdrawing substitutents on the pK_a values of the pigments and on the corresponding Schiff bases in solution were analyzed. The data suggest that the apparent pK_a of the protonated Schiff base is above 16. However, the alternative possibility that the retinal Schiff base linkage in bovine rhodopsin is not accessible for titration from the aqueous bulk medium cannot be definitely ruled out.

INTRODUCTION

The visual process is initiated via light absorption by visual pigments called rhodopsins. Rhodopsin (Rh) consists of an 11-cis retinyl polyene, 1, covalently bound to a membrane apo-protein (opsin) via a protonated Schiff base linkage with a lysine residue (for reviews, see references 1-5). Rhodopsin excitation ultimately leads to changes in the electrical potential of the photoreceptor cell membranes which are transmitted to the brain through appropriate synaptic processes.

Absorption of a photon by rhodopsin initiates a series of thermal reactions resulting in deprotonation of the Schiff base, forming a blue-shifted metarhodopsin II (MII) intermediate. Studies with a rhodopsin bearing a methylated lysine residue (6), as well as with artificial pigments (7) suggest that the deprotonation process and formation of the MII intermediate are crucial for the biological activity. Obviously, determination of the pK_a of the protonated Schiff base is of primary importance for understanding the function of rhodopsin on a molecular level.

The spectrum of bovine rhodopsin was reported (8, 9) to be independent of pH in the pH range of 3-9. A recent paper (10) demonstrated that a high pH form of Rh absorbs at 494 nm (relative to 500 nm at pH = 7). The shift was attributed to deprotonation of a single amino acid with a pK_a of 10.2. However, deprotonation of the Schiff base was not observed, at least up to pH = 11, when progressive partial protein denaturation precludes further pH increment. In contrast, the retinal protonated Schiff base was titrated with a pK_a = 6 in a bovine rhodopsin mutant in which a single residue (Glu 113) was replaced by glutamine (11-13). The reason for the large pK_a change of the protonated Schiff base, following a single carboxylic acid mutation, as well as its variation with the type of acid used, are not fully understood.

In contrast to the failure to titrate the Schiff base of native visual rhodopsin, that of the closely related photosynthetic pigment bacteriorhodopsin (bR) is (partially) titrable, exhibiting a pK_a of 13.3 ± 0.3 (14). Moreover, we have been able to show that the pK_a of bR may be controlled by modifying the intrinsic pK_a of the retinal by using fluorinated synthetic chromophores (15). This approach is now applied to a visual pigment. Our results suggest that the apparent pK_a of the protonated Schiff base of bovine rhodopsin is above 16. However, the possibility that in bovine rhodopsin, unlike bacteriorhodopsin, the Schiff base linkage is not accessible for titration from the aqueous bulk medium cannot be ruled out. We also conclude that although the exact apparent pK_a value of rhodopsin could not be determined, its value must exceed that (7.3 ± 0.1) of a model retinal Schiff base in solution.

MATERIALS AND METHODS

Chromophores 2–4 were prepared by previously described methods (16-18). Chromophore 5 was prepared by condensation of 9-*cis* β-ionylidenealdehyde (C_{15} aldehyde prepared from β-ionone) with 1,3-dichloroacetone in the presence of three equivalents of piperidine and acetic acid in THF at 0°C for 1 h. Wittig-Horner reaction with the sodium salt of triethylphosphonoacetate, followed by reduction with diisobutylaluminium hydride at −70°C and oxidation with MnO_2 afforded chromophore 5.

^{1}H NMR: $CDCl_3$ δ 1.06 (s, 6, C_{16}—H, C_{17}—H), 1.77 (s, 3, C_{18}—H), 2.15 (s, 3, C_{19}—H), 4.77 (s, 2, C_{20}—H), 6.68 (d, J = 16, 1, C_7—H), 6.49 (d, J = 16, 1, C_8—H), 6.63 (d, J = 7, 1, C_{14}—H), 6.52 (d, J = 11.3, 1, C_{10}—H), 7.50 (d, J = 12, 1, C_{11}—H), 10.14 (d, J = 7, 1, C_{15}—H).

The Schiff bases of aldehydes 2, 3, and 5 were prepared by dissolving the aldehyde in dry ethanol and mixing the solution with 2 equivalents of *n*-butylamine at 25°C for 30 min in the dark. Evaporation of the solvent and excess *n*-butylamine under reduced pressure afforded the corresponding Schiff bases. The Schiff base of chromophore 4 was prepared similarly but in methylene chloride instead of ethanol, due to the instability of the chromophore in ethanol.

Titrations of the Schiff bases were carried out under a dim red light by recording the absorption maxima of 1:1 ethanol-water solution at 0°C in the presence of appropriate buffers. The absorption maxima were measured using a Hewlett Packard Co. (Palo Alto, CA) 8450A diode array spectrophotometer and at 0°C to prevent decomposition of the chromophores. The apparent pK_a values were determined by recording the change in the intensity of the protonated Schiff base band and that of the corresponding deprotonated form.

The absorption maxima of the protonated Schiff base in ethanol were recorded by Schiff base titration with ethanol solution saturated with HCl.

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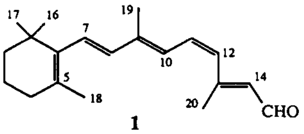
TABLE 1 Absorption maxima (λ_{max}) of the free chromophores and their corresponding pigments

Chromophore	λ_{max}, nm Aldehyde	SB	SBH^+	Pigment λ_{max}	SBH^+ pK_a
				nm	
1	380	360	440	500	7.4 ± 0.1
2	384	370	460	522	5.0 ± 0.1
3	388	372	460	510	5.0 ± 0.1
4	400	367	467	512	1.8 ± 0.1
5	402	370	454	520	3.8 ± 0.1

SB, unprotonated Schiff base; SBH^+, protonated Schiff base in methanol.



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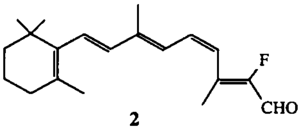

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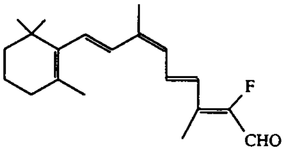

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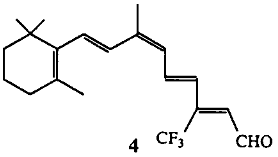

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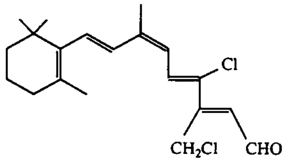

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The artificial bovine rhodopsin pigments were prepared from bovine opsin, following a previously described procedure (19). Titrations were carried out by mixing the pigment with appropriate buffer and monitoring the pigment absorption.

RESULTS AND DISCUSSION

The absorption maxima of chromophores 2, 3, 4, and 5 and their protonated Schiff bases are red shifted relative to 9-*cis* and 11-*cis* retinals (1) and their protonated Schiff base derivatives (Table 1). The shift originates from the electron-withdrawing character of the fluoro and the chloro groups, which probably destabilizes the positively charged ground state relative to the excited state. In the excited state, this destabilization effect is reduced due to positive charge migration towards the ring moiety.

Destabilization of the ground state due to the halogeno substitutents affects the pK_a of the chromophores as well. Thus, the apparent pK_a value of both isomers, 2 (11-*cis*) and 3 (9-*cis*), is $pK_a = 5.0 \pm 0.1$ relative to $pK_a = 7.3 \pm 0.1$ of 1, whereas the pK_a of 4 is further shifted to $pK_a = 1.8 \pm 0.1$ and that of 5 is shifted to $pK_a = 3.8 \pm 0.1$.

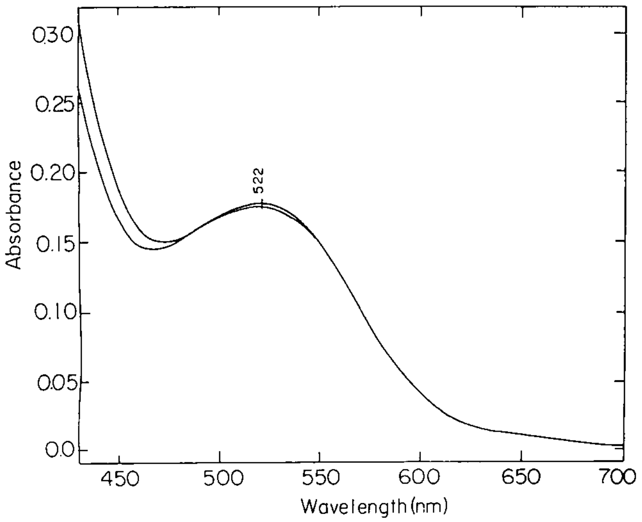

FIGURE 1 Absorption maximum of 14-F rhodopsin (II) at: pH = 7 and pH = 11.

Incubation of chromophores 2–4 with bovine opsin yielded three pigments absorbing at 522 nm, 510 nm (II and III), and 512 nm (IV) (Table 1) as previously described (20, 21). Incubation of 5 yielded a pigment which absorbed at 520 nm. The red shifts observed in the protonated Schiff base derivatives of the corresponding chromophores in solution relative to protonated retinal Schiff base, due to the halogeno groups, are also reflected in their pigments. This observation indicates that similar chromophore interactions prevail in bovine rhodopsin and in the artificial pigments. An attempt to titrate the three pigments by elevating the pH as high as pH = 11 did not reveal any detectable change in their spectra (Figs. 1–4). For pigment IV, the insensitivity to the pH change was further checked by comparing the amount of bleached pigment following exposure to light at pH 7

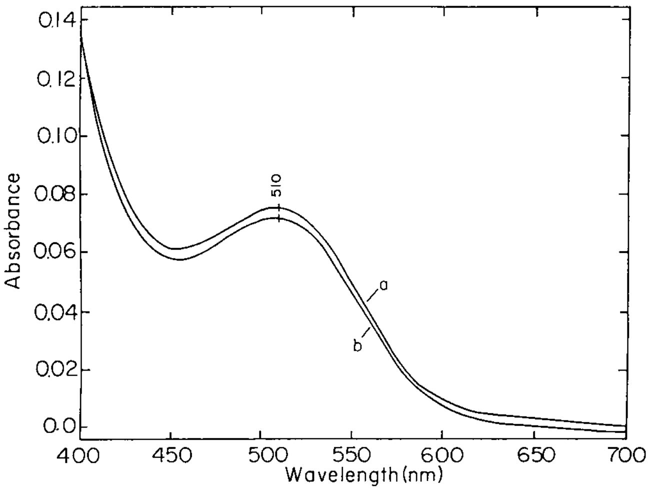

FIGURE 2 Absorption maximum of 14-F rhodopsin (III) at: (*a*) pH = 7; (*b*) pH = 11.

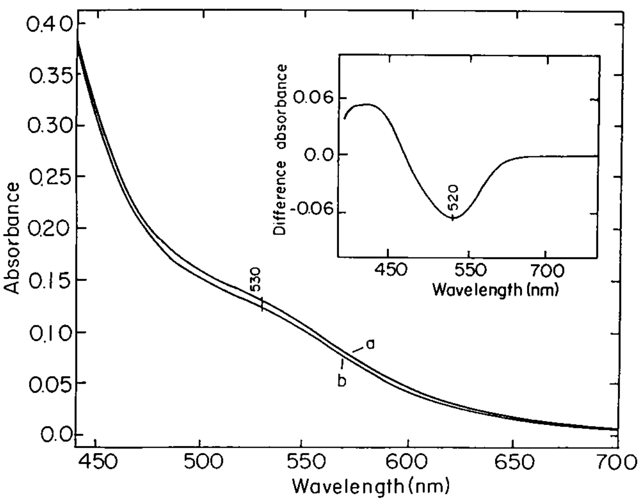

FIGURE 3 Absorption maximum of 20,20,20 trifluoroisorhodopsin (IV) at: (*a*) pH = 7; (*b*) pH = 11. (*Inset*) Difference spectra before and after pigment bleaching by white light. Similar difference spectra were obtained at pH = 7 and pH = 11.

and 11 (Fig. 3). Above pH 11, protein denaturation started to appear. Thus, despite the intrinsic reduction induced in the pK_a of the free retinal protonated Schiff bases (by 2.3 units in 2 and 3, by 5.5 units in 4, and by 3.5 units in 5) we did not observe any Schiff base deprotonation in the artificial rhodopsin derived from 2 or the artificial isorhodopsins (9-*cis*) derived from 3, 4, and 5. A basic feature of artificial bacteriorhodopsins containing fluorinated retinals with reduced pK_a values is the additivity of the intrinsic change in the pK_a of the retinal with that induced by the protein. Thus, the pK_a values of the protonated Schiff bases of synthetic retinals all-*trans* 20,20,20 trifluororetinal (the trans isomer of 4) and trans 14-F retinal are shifted by 5.5 and 2.3 units with respect to the all-*trans* retinal. Analogous shifts, 5.3 and 2, respectively, are observed between the corresponding pigments and native bR (reference 15 and Sheves et al., unpublished results). It is unlikely that special protein-chromophore interactions in bovine rhodopsin completely abolish the effect of fluoro and chloro groups on the intrinsic pK_a of the retinal chromophore. This conclusion is based (as mentioned above) on the observed normal red shifts that the synthetic retinal analogues experience following formation of the pigment. Thus, significantly reduced pK_a values are expected in the artificial pigments due to the substitution of fluoro and chloro groups.

On this basis, our present observations imply that either the apparent pK_a of the protonated Schiff base in bovine rhodopsin is higher than ~16 or that the Schiff base linkage is not accessible for titration from the bulk medium. Direct evidence showing the penetration of OH^- or H^+ ions to the rhodopsin binding site is not available. However, it appears that water penetrates the binding site of bovine rhodopsin quite rapidly. Thus, it was recently shown (R. Callender et al. manuscript in preparation) that the hydrogen-deuterium exchange time of the protonated Schiff base is of the order of milliseconds. The observation (10) that it is possible to shift the absorption maxima of bovine rhodopsin by titrating an amino acid (with a pK_a of 10.2) may also be indicative of the accessibility of the rhodopsin binding site to protons. These arguments tend to favor the possibility that the apparent pK_a of the protonated Schiff base of bovine rhodopsin is higher than 16. However, additional experimental data on the exposure of the binding site to the external aqueous medium will be required for excluding the alternative explanation of our data, namely, that the Schiff base linkage is not accessible for titration.

As mentioned above, analogous experiments carried out with the artificial bacteriorhodopsin (bR) pigment derived from the all-trans isomer of chromophore 4 revealed that the intrinsic change induced in the pK_a of the free retinal Schiff base was faithfully reflected in the bacteriorhodopsin pigment. On the basis of this observation, it was concluded that in bR the protonated Schiff base is accessible for titration from the bulk medium and that its apparent pK_a is 13.3 ± 0.3. Thus, independently of the specific interpretation of our observations with the fluorinated chromophores, it is evident that the retinal binding sites of bovine rhodopsin and bacteriorhodopsin are substantially different.

The fact that even chromophore 4, in which its intrinsic protonated Schiff base pK_a was reduced by 5.5 units (from 7.4 ± 0.1 to 1.8 ± 0.1) still formed a pigment with opsin, is in keeping with significant elevation of the apparent pK_a of the protonated Schiff base in bovine rhodopsin (relative to the solution value). Following light absorption, this pK_a is reduced and deprotonation takes place to form the MII intermediate. In this respect, it is interesting to note that a single substitution of Glu113 by glutamine in bovine rhodopsin modifies the apparent pK_a of the retinal protonated Schiff base to ~6 (11–13).

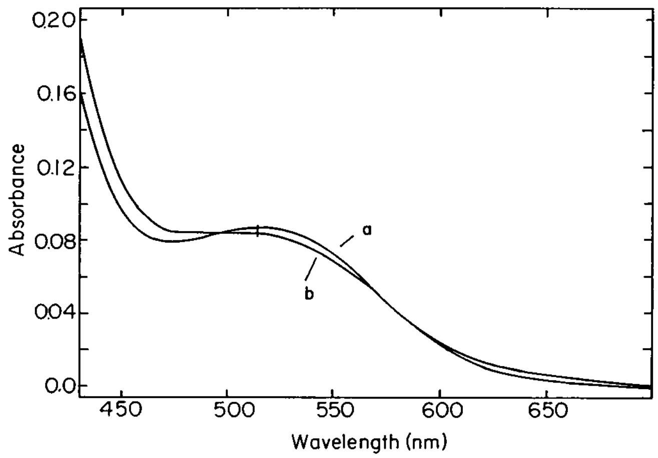

FIGURE 4 Absorption at pH = 7 (*a*) and pH = 11 (*b*) of 12-Cl, 20-Cl isorhodopsin (V).

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Our present studies suggest that either this substitution enables accessibility of the Schiff base to titration, probably due to a protein conformational change, or that the mutation causes a dramatic change in the pK_a. If indeed the Schiff base in bovine rhodopsin is titrable, then accounting for the value of $pK_a > 16$ is a difficult task. There are a few mechanisms for raising the pK_a of the protonated Schiff base. Besides the distance between the positively charged polyene and its carboxylate counterion, hydrogen bonding to the carboxylate and the Schiff base by polar groups significantly affect the pK_a (22) and can account for other spectroscopic properties of rhodopsin (23). Recent studies with model compounds (24) indicate that a certain defined angle between the protonated retinal Schiff base and the carboxylate group, which allows for water to bridge the two groups and to form effective hydrogen bonding with the carboxylate group, can raise the apparent pK_a of protonated retinal Schiff base significantly (by ~5.5 units). This experiment suggests that the significant reduction of the pK_a of bovine rhodopsin by the single substitution of Glu 113 may be due to perturbation of the special carboxylate/water/Schiff base structure. Still, future studies should be performed to confirm and to account for the suggested value of $pK_a > 16$ in rhodopsin.

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