brief communication

# High-pH form of bovine rhodopsin

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ABSTRACT Since the 1930s, the spectrum of vertebrate rhodopsin has been considered to be independent of pH (Lythgoe, R.J. 1937. *J. Physiol.* 89:331–358; Wald, G. 1938. *J. Gen. Physiol.* 21:795–832). Here I report that the spectrum of bovine rhodopsin is pH dependent. At pHs > 9.0, there is a shift to shorter wavelengths of its 500-nm absorption band. This shift is accounted for by the existence of a high pH form of bovine rhodopsin, with absorption maximum at 494 nm and a slightly lower extinction coefficient. The high-pH form results from the low-pH form by the deprotonation of a single group with a pK of ~10.2 for rhodopsin in rod disk membranes in 4.0 M KCl. The shift is observed for sheep and chicken rhodopsins, but not for frog, toad, and octopus rhodopsins. This indicates a specific amino acid difference between these rhodopsins that is potentially relevant for the mechanism of color regulation.

## INTRODUCTION

Rhodopsin, an integral membrane protein, is the light-sensitive pigment of the photoreceptors of higher organisms. Light absorption induces protein conformational changes that trigger an enzymatic cascade resulting in the electrical excitation of the photoreceptor cell (Stryer, 1986; Koutalos and Ebrey, 1986; Pugh and Cobbs, 1986; Tsuda, 1987). Rhodopsin consists of a light-sensitive chromophore, which for almost all rhodopsins is 11-*cis* retinal, and a light-insensitive protein moiety, opsin. Rhodopsins from different species have also different spectral, photochemical, and enzymatic properties; because the chromophoric group is the same, the varying properties are considered to arise from differences in the protein part and its interaction with retinal. Therefore, the studying of the opsin-chromophore interaction is of primary importance for understanding the physiologically relevant properties of rhodopsin.

The pH dependence of the rhodopsin spectrum is one way of probing the opsin-chromophore interactions. The spectra of rhodopsin photoproducts have been known to be pH dependent (Lythgoe, 1937; Wald, 1938; Matthews et al., 1963). The spectrum of vertebrate rhodopsin itself has been reported to be independent of pH (Lythgoe, 1937; Wald, 1938), though slight changes with pH have been reported for the spectrum of frog rhodopsin (Chaise and Haig, 1938).

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## MATERIALS AND METHODS

Bovine rod outer segment membranes were prepared from frozen retinas (Lawson Co., Lincoln, Nebraska) according to the method of Papermaster and Dreyer (1974). The 280/500 ratio was 2.4–2.5. Sheep eyeballs were obtained from the University of Illinois slaughterhouse, whereas chicken eyeballs were obtained from Professor J. Bahr's laboratory at the University of Illinois, Urbana, Illinois; chickens were killed by an injection of a sodium pentobarbital solution. Frogs (*Rana pipiens,* from Kons Scientific Co., Germantown, Wisconsin) and toads (*Bufo marinus,* from National Reagent, Bridgeport, Connecticut) were killed under dim red light by decapitation and double-pithing. The retinas of frog, toad, and sheep were removed from the eyes under dim red light. Frog rod outer segment membranes were prepared by the same method as for bovine. Sheep and toad rod outer segment membranes were prepared by homogenization of the retinas and wash in 10 mM tris acetate buffer, pH-7.4, a flotation in 40% sucrose in the same buffer, and two further washes in the same buffer. Chicken photoreceptor membranes were prepared by the method of Chen et al. (1989). After two washes in 400 mM KCl, the membranes were suspended in 4.0 M KCl and used for the pH titrations. For spectral measurements, quartz cuvettes with 2-ml samples were used in an Aviv 14DS spectrophotometer having an end on photomultiplier. The pH was adjusted by additions of small amounts of KOH, and the pH was measured with a Beckman open glass electrode; each spectrum was corrected for dilution. Throughout the measurements, the sample was magnetically stirred. The room temperature was 23°C. The spectra were recorded from 800 to 330 nm, and corrected for scattering by the subtraction of the spectrum of a Sephadex-100 solution, so that the spectrum was flat in the 650–800-nm region. The same results were obtained when the spectrum used for correction was the one of bleached or denatured sample at high pH (> 12), after the end of the titration.

## RESULTS AND DISCUSSION

Fig. 1 *a* shows spectra of bovine rhodopsin in membranes at different pHs after correction for scattering. There is a shift of the absorption maximum to shorter


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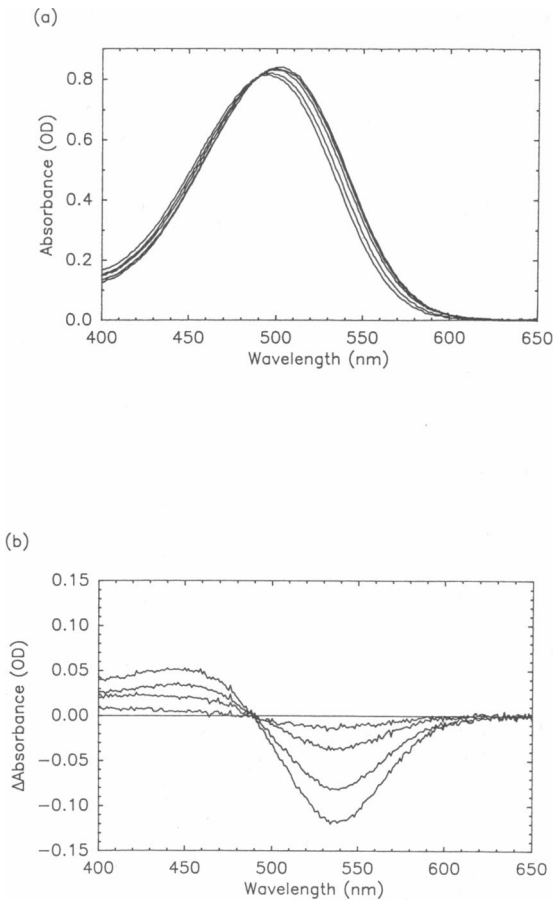


FIGURE 1 (*a*) Spectra from bovine rhodopsin in membranes in 4.0 M KCl. The pHs are, with the band shifting to shorter wavelengths: 6.58, 9.33, 10.04, 10.53, and 11.15; (*b*) Difference spectra from (*a*) above: reference spectrum at pH-6.58 (zero line), the rest in the order of decreasing absorbance at 540 nm are at pHs 9.33, 10.04, 10.53, and 11.15.

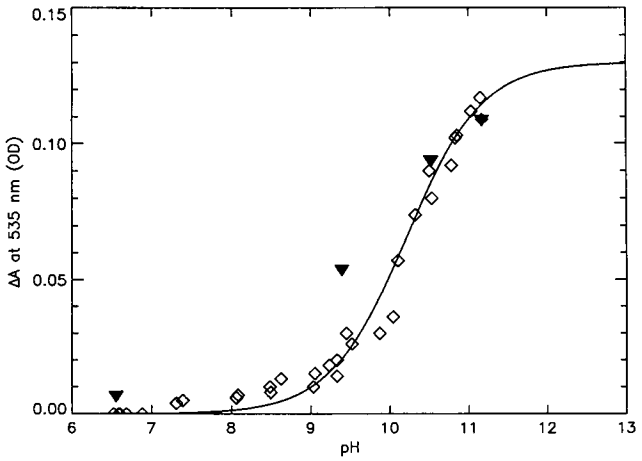


FIGURE 2 Titration points from difference spectra as the ones in Fig. 1 *b*. Absorption difference at 535 nm is plotted versus pH. Increasing pHs: (◇); and decreasing pHs: (▼). The continuous curve is fitted through the points for increasing pHs, and has a pK of 10.2, a 0.9 number of protons, and a maximum of 0.13 o.d. for the total change at 535 nm for going from rhodopsin to alkaline rhodopsin.

wavelengths, accompanied by a slight drop in absorption as the pH gets higher. Fig. 1 *b* shows the difference spectra for the transition. The absorption minimum for each difference spectrum is at 537 nm, indicating that this transition is quite different from the alkaline denaturation of rhodopsin (difference spectrum minimum at 500 nm), which occurs rapidly at higher pHs ($> \sim 11.4$, see below). Fig. 2 shows the titration data for the transition. The titration curve which has been fitted through the points has a pK of 10.2 and involves 0.9 protons. The transition is reversible, though a hysteresis is evident in the graph. The reversibility of the transition indicates that within the time scale of the experiment no significant denaturation is occuring at these high pHs. Other spectra showed that, under my conditions, no more than 3% of total rhodopsin denatures in 30 min at a pH of 10.9 (data not shown).

The bandshift at high pH is attributed to the presence of a high-pH form of bovine rhodopsin, which will be referred to as alkaline rhodopsin; the name rhodopsin has been kept for the usual neutral pH form. Because the titrations have been up to 90% complete (see Fig. 2), the spectrum of alkaline rhodopsin is reconstructed on the basis of the titration curve. The spectrum is shown in Fig. 3, along with rhodopsin's spectrum for comparison. Alkaline rhodopsin has an absorption maximum at 494 nm and its band is slightly broader (by 200 $cm^{-1}$) than

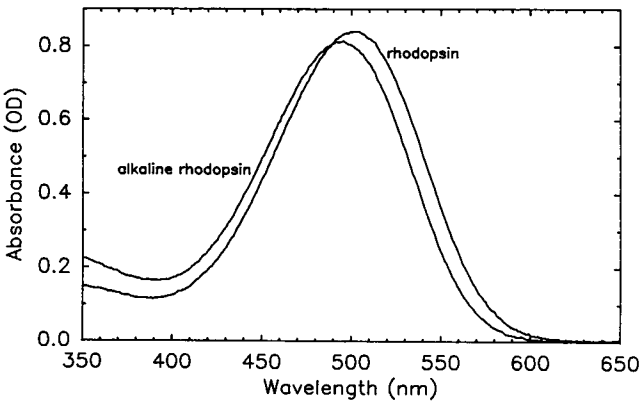


FIGURE 3 Alkaline rhodopsin and rhodopsin spectra, plotted to scale.

rhodopsin's, in quantitative agreement with the empirical relationship between absorption maximum and bandwidth for rhodopsins (Ebrey and Honig, 1977). Table 1 compares the spectral parameters for alkaline rhodopsin and rhodopsin, as measured in the present study.

To ascertain that the observed bandshift is due to rhodopsin and not to another property of the preparation the following, somewhat overlapping properties for the transition were established (data not shown):

(*a*) It disappears upon the bleaching of rhodopsin, therefore it is associated with a light-sensitive component.

(*b*) It is unaffected by the addition of reducing agents (sodium bisulfite), and therefore is not due to cytochrome contamination.

(*c*) It is not due to the alkaline denaturation of a cone pigment. This was shown by the failure to regenerate any cone pigment by addition of 11-*cis* retinal, as well as by the virtual identity of the successive bleaching difference spectra, through a series of cut-off filters.

(*d*) In connection with the above, the relatively large size of the absorption changes characterizing the transition (15% of total rhodopsin, compare the spectra of Fig. 1 *a* and *b*) suggests that they are due to rhodopsin.

(*e*) The transition is reproducible with rod outer segment membranes prepared from different batches of retinas, prepared according to different methods (Papermaster and Dreyer, 1974; Fung and Stryer, 1980), and with varying degrees of cytochrome contamination. Moreover, the amount of alkaline rhodopsin present in each sample (as calculated from the spectra and the titration curves) correlates with a coefficient higher than 99% with the amount of rhodopsin initially present.

(*f*) The transition is also observed in detergent-solubilized rhodopsin (in 1% CHAPSO, as well as in 1% Octylglucoside). But, the rapid denaturation of detergent-solubilized rhodopsin at high pHs did not permit me to carry out the same extensive study of the transition in detergent as in membranes.

The significance of the described transition lies in its function as an indicator of a specific protein-chromophore interaction. The same transition is observed for sheep rhodopsin in 4.0 M KCl, and a similar spectral shift is observed for chicken rhodopsin in 1% Octylglucoside over the same pH region (data not shown; it was not possible to obtain spectra from chicken photoreceptor membranes, because of the high amount of scattering). No such transition or spectral bandshift is observed with octopus rhodopsin (Koutalos et al., 1990; Koutalos and Ebrey, unpublished observations), or with frog or toad rhodopsins. This suggests an important difference between frog and toad, and bovine, sheep and chicken rhodopsins (all vertebrates), as well as between octopus and bovine rhodopsins. The number of protons involved indicates that the deprotonation of a single amino acid group is responsible for the transition. The pK of 10.2 (in 4.0 M KCl) is consistent with the deprotonation of a tyrosine, cysteine, lysine, or another amino acid, whose pK is shifted by the protein environment. In the case of bacteriorhodopsin, a bacterial retinal-containing protein, a tyrosine deprotonation has been implicated in a 1.5-nm red shift of the absorption band at high pHs (Balashov et al., 1991). In the case of rhodopsin, the spectral shift due to the deprotonation is larger (7 nm, or 240 $cm^{-1}$) and to the blue. The shift can be accounted for by a change in the protein-chromophore interaction caused by the deprotonation and mediated either by an electrostatic interaction or by a conformational change. Because aromatic amino acid side chains have been proposed to interact with the retinal's polyene chain (Rafferty, 1979; Kakitani et al., 1985), another possible explanation for the spectral shift is the perturbation by the deprotonation of the interaction of a tyrosine aromatic side chain with the chromophore. Most of the amino acids potentially responsible for the transition are conserved in the bovine, sheep, and chicken rhodopsins (Hargrave et al., 1983; Pappin et al., 1984; Takao et al., 1988), whereas the single group differences between bovine and octopus rhodopsins (Hargrave et al., 1983; Ovchinnikov et al., 1988) are too great to be able to say anything more on the group's identity. Frog and toad rhodopsins do not show the spectral band shift and would be expected to lack the responsible amino acid. But, their sequences are not known to compare them with the sequence of bovine rhodopsin.

**TABLE 1 Spectral properties of rhodopsin and alkaline rhodopsin**

| Spectral property | Rhodopsin | Alkaline rhodopsin |
|---|---|---|
| Absorption maximum (nm) | 501 ± 1 | 494 ± 1 |
| Bandwidth (1/cm) | 4,000 ± 100 | 4,200 ± 100 |
| Relative extinction at maximum | 1.00 | 0.97 ± 0.01 |

The difference spectrum absorption maximum is at 537 ± 2 nm; the isosbestic point is at 490 ± 2 nm.

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