brief communication

Factors affecting the absorption maxima of acidic forms of bacteriorhodopsin

A study with artificial pigments

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ABSTRACT The absorption maximum (568 nm) of light-adapted bacteriorhodopsin bR_{568} undergoes reversible changes after acidification. At pH 2.9, the absorption shifts to 605 nm (forming bR_{605}) and it blue shifts to 565 nm, after further acidification to pH ~ 0.5 (forming bR_{565}). Molecular models accounting for such acid-induced changes are relevant to the structure and function of bacteriorhodopsin. In the present study we approached the problem by applying artificial bR pigments based on selectively modified synthetic retinals. This may allow direct identification of the specific regions in the retinal binding site where the above changes in the protein-retinal interactions take place. We investigated the spectroscopic effects of acid in a variety of artificial pigments, including cyaninelike retinals, retinals bearing bulky groups at C_4, short polyenes, and retinals in which the β-ionone ring was substituted by aromatic rings. The results provide direct evidence for the hypothesis that the generation of bR_{605} is due to changes in polyene-opsin interactions in the vicinity of the Schiff base linkage. The second transition (to bR_{565}) was not observed in artificial pigments bearing major changes in the ring structure of the retinal. Two approaches accounting for this observation are presented. One argues that the generation of bR_{565} is associated with acid-induced changes in retinal-protein interactions in the vicinity of the retinal ring. The second involves changes in polyene-opsin interactions in the vicinity of the Schiff base linkage. For both bR_{605} and bR_{565}, our results do not discriminate between the direct titration of negative or dipolar protein groups in the binding site and changes in the retinal-protein interactions induced by changes in the protein structure outside of the binding site.

INTRODUCTION

Light-adapted bacteriorhodopsin (bR_{568}), the all-*trans* retinal-protein complex in the purple membrane of *Halobacterium halobium,* functions as a light-driven proton pump (for review see Stoeckenius and Bogomolni, 1982). The function of bR is based on the characteristic absorption spectrum of the pigment (λ_{max} = 568 nm) and on its cyclic photoreaction. Both are closely associated with specific electrostatic and steric interactions between the retinyl polyene (chromophore I in Table 1) and its protein environment, to which it is linked via a protonated Schiff base bond.

Acidification of purple membrane suspensions induces reversible transitions of bR_{568} into two forms: one absorbing 605 nm (bR_{605}) with an apparent pK_a of 2.9, and a second absorbing at 565 nm (bR_{565}) with pK_a = 0.5 (Oesterhelt and Stoeckenius, 1971; Fischer and Oesterhelt, 1979). Later studies (Kimura et al., 1984; Chang et al., 1985) have shown that the

$$bR_{568} \overset{H^+}{\rightleftharpoons} bR_{605}$$

equilibrium is markedly affected by deionization of the membrane suspensions. Thus, when bR-bound divalent cations are removed, the equilibrium shifts to bR_{605} even in neutral solutions. On the other hand, the

$$bR_{605} \overset{H^+, Cl^-}{\rightleftharpoons} bR_{565}$$

equilibrium is unaffected by deionization.

Considerable interest in bR_{605} and bR_{565}, as clues to the structure and function of bR, arose in relation to several points. (*a*) The absorption spectrum. Based on indirect evidence, it was suggested that the formation of bR_{605} is associated with acid-induced changes in the interactions between the Schiff base and its counterion. It was proposed that such changes are induced either by direct titration (neutralization) of the intrinsic counterion (Fischer and Oesterhelt, 1979; Warshel and Ottolenghi, 1979; Smith and Mathies, 1985), or by a protein conformational change inducing an increased Schiff base-counterion separation (Fischer and Oesterhelt, 1979; Smith and Mathies, 1985; Szundi and Stoeckenius, 1987, 1988). The generation of bR_{565} was attributed to protonation of a protein group in the vicinity of the β-ionone ring (Warshel and Ottolenghi, 1979), or to binding of an extrinsic anion at the site responsible for the first transition (Fischer and Oesterhelt, 1979). More recently it has been suggested that low pH and/or high extrinsic ion

TABLE 1 Absorption maxima of artificial pigment at different pH

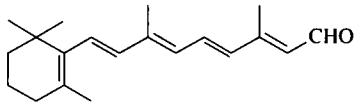
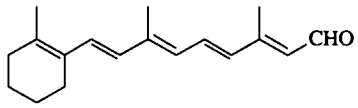
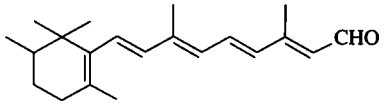
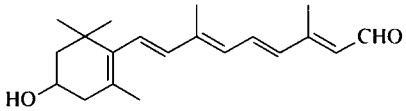
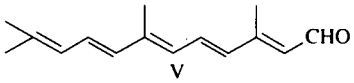
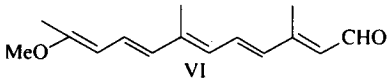
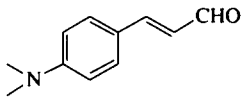
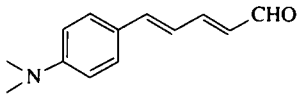
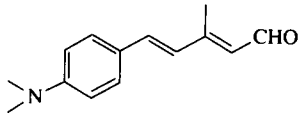
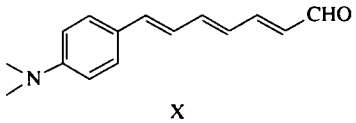
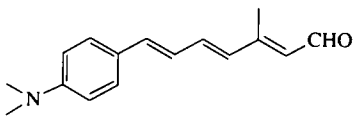
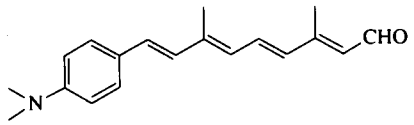
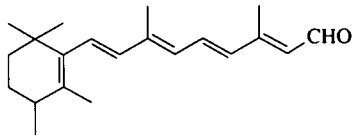
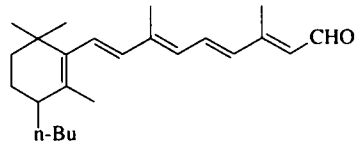
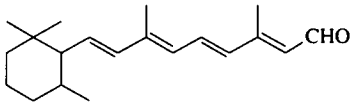
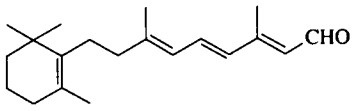
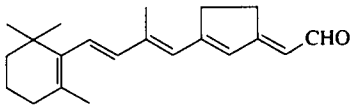
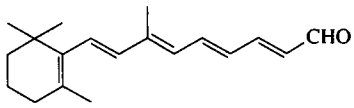
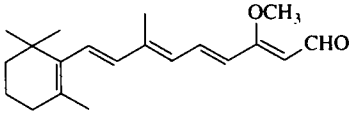
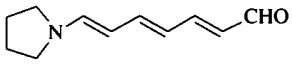
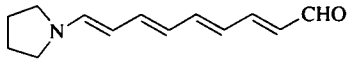
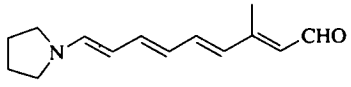
Pigment	Absorption maxima			Spectral shifts	
	pH7	pH2.5 (bR_i^1)	pH ~0.5 (bR_i^2)	$\Delta\nu_1$*	$\Delta\nu_2$†
	nm	*nm*	*nm*	cm^{-1}	cm^{-1}
 I	570	605	565	1015	1170
 II	552	592	560	1225	960
 III	557	578	556	650	680
 IV	556	610	552	1590	1720
 V	527	558	533	875	840
 VI	572	615	590	1220	690
 VII	518	540	540	790	—
 VIII	582	630	634	1250	—
 IX	576	620	631	1230	—
 X	615	650	650	870	—
 XI	590	635	635	1180	—

TABLE 1 (continued)

 XII	570	611	618	1180	—
 XIII	462	494	505	1400	—
 XIV	469	503	510	1450	—
 XV	466	484	484	800	—
 XVI	440	455	455	550	—
 XVII	524	560	560	1225	—
 XVIII	554	595	595	1250	—
 XIX	520	552[‡]	544	1000	—
 XX	529	529	529	—	—
 XXI	640	640	640	—	—
 XXII	665	662	661	—	—

*Difference in energy between absorption maxima at pH 7 and pH ~2.5 (first acid transition).
†Difference in energy between absorption maxima at pH ~0.5 and pH ~2.5 (second acid transition).
‡The absorption band changes after ~60 s to a band at 450 nm. See also Gartner and Oesterhelt, 1988.

concentrations induce the formation of bR_{565} by restoring a Schiff base environment similar to that of the native bR_{568} chromophore (Szundi and Stoeckenius, 1987, 1988). (*b*) The photocycles. Both bR_{605} (Mowery et al., 1979; Kobayashi et al., 1983; Dupuis et al., 1985; Chang et al., 1985; Ohtani et al., 1986) and bR_{565} (Mowery et al., 1979) exhibit photocycles which differ from that of bR_{568}. Especially relevant is the observation that bR_{605} lacks the characteristic M_{412} photointermediate of bR_{568} and does not pump protons. (*c*) Cation binding in bR. Binding sites and binding mechanisms and their relationships with the membrane surface potential, with the protein conformation, and with photodeprotonation of the Schiff base, are all closely associated with the $bR_{568} \rightleftharpoons bR_{605}$ equilibrium (Chang et al., 1986; Dunach et al., 1987; Concoran et al., 1987; Szundi and Stoeckenius, 1987, 1988; Ariki et al. 1987). (*d*) The direction of the exposure of the Schiff base in respect to the membrane surface (Druckmann et al., 1985). (*e*) The kinetic mechanism of light → dark (all-*trans* → 13-*cis*) adaptation (Warshel and Ottolenghi, 1979).

In spite of such extensive activity, the molecular changes in the protein and in the protein-retinal interactions responsible for the generation of bR_{605} and bR_{565} are still unclear. In the present work we approach the problem by applying artificial bR pigments in which the native retinal has been replaced by synthetic analogues (for reviews see Derguini and Nakanishi, 1986; Sheves et al., 1987). The method has substantially contributed to the understanding of the color and photocycle of bR. It now bears on the identification of specific regions in the polyene-binding site, associated with the formation of the acid forms of bacteriorhodopsin.

MATERIALS AND METHODS

The synthesis of the retinal analogues has been described previously (for review see Ottolenghi and Sheves, in press. For pigment XVIII see Gartner et al., 1983; for XIX see Gartner and Oesterhelt, 1988; for XX–XXII see Derguini et al., 1983; Sheves and Friedman, 1986). The artificial pigments were prepared by reconstituting the apomembrane suspended in water with the retinal analogues at 25°C (Tokunaga and Ebrey, 1978). The acidic forms of the artificial pigments were obtained by mixing the pigments with appropriate buffers. Absorption spectra were measured using a model 8450A diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA).

RESULTS AND DISCUSSION

The effects of acid on the absorption spectra of a variety of artificial light-adapted bR pigments are summarized in Table 1. The effects may be classified according to three main categories: (*a*) pigments II–VI which, analogously to bR_{568} (I), exhibit two spectral transitions: a red shift with an apparent $pK_a \sim 2.5$, followed by a blue shift with $pK_a \sim 0.5$. (*b*) pigments (VII–XIX), which exhibit only the first transition, and (*c*) pigments whose spectra are unaffected by acid down to pH ~0.5 (XX–XXII).

It is worthwhile noting that in most cases of categories *a* and *b*, the magnitude of the spectral shift associated with the first transition ($\Delta\nu_1 = 1{,}100 \pm 350$ cm^{-1}) is reasonably close to that characterizing the $bR_{568} \rightarrow bR_{605}$ interconversion in the native pigment ($\Delta\nu = 1{,}015$ cm^{-1}). Somehow higher positive or negative deviations from the latter value are observed in the cases of III, IV, and XVI. We thus conclude that with class *a* and *b* pigments we observe the generation of acid species (bR^1_{II}, bR^1_{III}, etc.) which are analogous to bR^1_7 (bR_{605}). The same applies in the case of class *a* to the parameter $\Delta\nu_2$. The latter measures the blue shift (1,200 ± 550 cm^{-1}) associated with the conversion of the first acid species to the highly acidic forms (bR^2_{II}, bR^2_{III}, etc.) which are thus assumed to be analogous to bR^2_1 (bR_{565}).

We first consider the formation of the bR^1_i species which bear on the structure of bR_{605}. These are observed even when the polyene chain is seriously perturbed, including the extreme case of VII in which only one C=C bond is present. This implies that the respective spectral shift must be induced by changes in protein-retinal interactions in the vicinity of the C_{13}-NH^+ region of the chromophore. A plausible specific mechanism, in keeping with previous suggestions (Warshel and Ottolenghi, 1979; Fischer and Oesterhelt, 1979) and with current models for the spectrum of bR (Lugtenburg et al., 1986; Spudich et al., 1986; Ottolenghi and Sheves, in press), may be based on the direct titration of a negative ion (counterion) in the vicinity of the protonated Schiff base. However, alternative (indirect) mechanisms associated with acid-induced changes in the conformation of the protein in the vicinity of Schiff base are also feasible. For example, an increased nitrogen counterion separation (Fischer and Oesterhelt, 1979; Smith and Mathies, 1985; Szundi and Stoeckenius, 1987, 1988), or changes in H-bonding to the nitrogen of residual water molecules or of other protein residues (Warshel and Barboy, 1982; Baasov and Sheves, 1986), may both be associated with a red spectral shift.

The only exceptions in respect to bR^1_i formation are the class *c*, cyaninelike pigments. Their insensitivity to acidification is readily accounted for by the basic insensitivity of the spectra of their parent (symmetric) cyanine dyes to nonconjugated charges, as well as to H-bonding to the NH^+ moiety (Sheves and Friedman, 1986)

Evidence supporting the conclusions that the generation of bR_{605} is due to a structural perturbation in the vicinity of the Schiff base linkage may also be derived from ^{13}C NMR studies of bR (Harbison et al., 1985) and of model protonated Schiff bases in solution (Albeck, A.,

H. Gottlieb, and M. Sheves, manuscript in preparation). A significant perturbation is observed in the $^{13}C_5$ chemical shift in bR (144.5 ppm), which is shifted by ~12 ppm downfield, relative to a model system (131 ppm). This dramatic effect on the chemical shift is partially due to the planar, *s-trans,* ring-chain conformation which prevails in the pigment. However, studies of model systems (Albeck, A., H. Gottlieb, and M. Sheves, manuscript in preparation) indicated that the $^{13}C_5$ chemical shift is also sensitive to changes in the Schiff base environment. Weakening of hydrogen bonding, which red shifts the spectrum, also affects the C_5 chemical shift. The effect is especially pronounced in the case of the *s-trans* ring-chain conformation. Model systems that mimicked the weakly H-bonded Schiff base of bR, exhibited a downfield shift of ~5 ppm in the C_5 chemical shift. Thus, assuming that the spectral shift associated with bR_{605} is due to perturbation of the Schiff base environment, such studies would predict an additional shift of 2–4 ppm for C_5 in bR_{605}. This is in keeping with the ~5 ppm value measured in bR_{605} by solid state ^{13}C NMR (de Groot et al., 1988).

We now consider the second acid-induced transition, from bR_i^1 (analogous to bR_{605}) to bR_i^2 (analogous to bR_{565}), observed in class *a* pigments in which the basic polyene system of bR_{568} is only slightly modified. Major changes in the ring region such as replacement by an aromatic ring (VII–XII), shortening the polyene chain (VII–XI) or of the polyene sequence (XV–XVI), and bulky substitution at the C_4 position (XIII–XIV) lead to elimination of the second transitions.

Molecular models accounting for these observations should be considered in light of the present approach to the spectrum of bacteriorhodopsin. The latter is quantitatively described by the "Opsin Shift" (OS) (Nakanishi et al., 1980), which measures the energy difference between the absorption maximum of a model protonated Schiff base in methanol solution (440 nm) and that of the pigment (568 nm). Accumulated evidence is now available (Baasov and Sheves, 1985; Harbison et al., 1985; Lugtenburg et al., 1986; Spudich et al., 1986) indicating that the observed value of OS in bacteriorhodopsin (~5,000 cm^{-1}) is the result of three major protein effects with contributions denoted by OS_s, OS_p, and OS_d. The first term accounts for more than half of the observed shift (i.e., OS_s ~ 3,000 cm^{-1}) and is attributed to electrostatic interactions involving the Schiff base nitrogen such as nitrogen-counterion separation, H-bonds, local dielectric factors, etc. The second term is attributed to a protein-induced planar ring-chain configuration (OS_p ~ 1,300 cm^{-1}) and the third to the presence of a protein ion pair (or a dipole) in the vicinity of the ring (OS_d ~ 700 cm^{-1}).

On the basis of this model for the binding site, two approaches, accounting for the $bR_{605} \rightarrow bR_{565}$ transition, may be advanced. The first is based on perturbation of the environment of the ring moiety. This can be carried out in various ways; e.g., Warshel and Ottolenghi (1979) suggested neutralization of a ring charge (ion pair) via direct titration. However, it has been suggested that the $bR_{605} \rightarrow bR_{565}$ transition is induced by extrinsic ions rather than by H^+ (Fischer and Oesterhelt, 1979; Kimura et al., 1984). In such a case, it will be more feasible to suggest that the effect is due to an increase in the polyene-ion pair distance due to an (indirect) acid-induced conformational change. Alternatively, it is possible that such a change induces a relaxation of the ring-chain conformation, from planar (*s-trans*) to distorted (*s-cis*), which will also result in a blue shift. Either of such alternatives will account for the lack of the second acid transition in artificial pigments bearing a seriously perturbated ring region. Moreover, it is expected that the value of $\Delta\nu_2$ for class *a* pigment will be comparable to OS_d or to OS_p, as it is actually confirmed by the data of Table 1. Because, $\Delta\nu_2$ is substantially smaller than $OS_p + OS_d$, the possibility that both acid-induced effects take place may be excluded.

The second approach interprets the spectrum of bR_{565} in terms of changes induced in the vicinity of the Schiff base linkage, restoring an environment similar to that of bR_{568}. Thus, the previous explanation, which excludes changes in the Schiff base environment as the source for the blue shift in bR_{565}, confronts difficulties with resonance-Raman measurements of acidified forms of bacteriorhodopsin (Smith and Mathies, 1985), indicating different C=N stretching frequencies for bR_{605} (1,630 cm^{-1}) and bR_{565} (1,637 cm^{-1}). This observation might reflect different isomer ratios (13-*cis* and all-*trans*) in both forms (Smith and Mathies, 1985) and/or a different Schiff base environment. For example, weak hydrogen bonding to the positively charged nitrogen with its counterion or with protein dipoles (or residual water) shifts the C=N frequency to a lower energy (Baasov et al., 1987; Rodman Gilson et al., 1988) and induces a red shift in the absorption spectrum. Thus, the change in the C=N stretching frequency in bR_{565} relative to bR_{605} might reflect stronger hydrogen bonding to the Schiff base linkage in the former, causing a blue shift in the spectrum. Obviously, in this case, the question arises as to why artificial pigments bearing a seriously perturbed ring region lack the second acid transition. A possible explanation implies an intimate coupling between the (tight) chromophore-protein conformation in the vicinity of the ring region and the change taking place in the Schiff base linkage environment in the second acid transition. Modification of the ring region in pigments VII–XVI perturbs the chromophore-protein interaction, preventing the change in the Schiff base environment in low pH.

The mechanisms suggested to account for the second

acid transition should also be considered in view of pigments XVII–XIX which, in spite of their basically unmodified polyene sequence, do not exhibit the transition to bR_i^2. We recall that such pigments differ from all others in Table 1 in carrying a 13-*cis*, rather than all-*trans*, chromophore (Gartner et al. 1983; Albeck et al. 1986). However, it appears that the 13-*cis* isomer of bR has a planar ring-chain conformation analogous to that of the all-*trans* isomer (Harbison et al., 1985). Thus, it is tempting to suggest that the 13-*cis* isomers lack the dipole-ring interactions which characterize analogous all-trans pigments and, therefore, do not exhibit transition to bR_i^2-like species. This interpretation will also qualitatively account for the decreased opsin shift (500–1,500 cm^{-1}) of the above 13-*cis* isomers in respect to analogous all-*trans* chromophores, such as I–IV. An alternative explanation for the observation that the 13-*cis* analogues lack the bR_i^2 transition may be associated with the finding that the $bR_{605} \rightarrow bR_{565}$ transition is coupled with a 13-*cis* → all-*trans* isomerization of the chromophore (Mowery et al., 1979, Smith and Mathies, 1985). Because chromophores that are *a priori* 13-*cis* (in both bR_{568} and bR_{605}) are unable to undergo such an isomerization, they will not generate the bR_i^2 species.

CONCLUSIONS

The application of artificial bR pigments provides direct experimental evidence indicating that the acid (or deionized) bR_{605} species is associated with changes in protein-polyene interactions in the vicinity of the Schiff base. The data are in keeping with the direct titration of a negative or dipolar protein group. Alternatively, titration of a protein group outside of the retinal binding site is also feasible. In such a case it is implied that the titration induces changes in the protein conformation that affect protein-retinal interactions in the vicinity of the Schiff base linkage. No definite conclusions can be derived in relation to the structure of the bR_{565} species. Thus, our data cannot discriminate between a mechanism based on changes in the protein environment around the β-ionone ring and one invoking changes in the vicinity of the Schiff base. If the latter applies, it is implied that such changes are conditioned by an intact chromophore structure in the ring region.

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