CHROMOPHORE/PROTEIN INTERACTION IN BACTERIAL SENSORY RHODOPSIN AND BACTERIORHODOPSIN

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ABSTRACT Retinal analogues with altered conjugated double bond systems or altered stereochemistry were incorporated into the phototaxis receptor sensory rhodopsin (SR) and the light-driven proton pump bacteriorhodopsin (BR) from *Halobacterium halobium*. Wavelength shifts in absorption ("opsin shifts") due to analogue interaction with the protein microenvironment demonstrate that the same overall electrostatic and steric properties of the retinal binding-site structures exist in both proteins despite their different functions. π-Electron calculations from the opsin shifts lead to a new description of protein charge distribution that applies to the binding sites of both SR and BR. The new data extends the previously proposed external point charge model for BR to include an ion-pair protein/chromophore interaction near the β-ionone moiety. The new data modifies the previously proposed external point-charge model, the derivation of which involved an experimentally erroneous opsin shift for one of the BR analogues.

INTRODUCTION

The chromophore of visual pigments and bacterial rhodopsins contain vitamin A aldehyde (retinal) covalently linked to a protein moiety (opsin). The retinal buried in the opsin interior and the protein structure forming the retinal pocket carries out the light transduction process: the conversion of photon absorption into protein conformational change. Accordingly, the interaction of retinal with opsin proteins has been the focus of both experimental and theoretical analyses (1, 2, 3). The binding of various synthetic analogues to the apoproteins has provided a versatile tool to clarify the properties of these pigments (4).

Bovine rhodopsin is a eucaryotic sensory receptor, whereas bacteriorhodopsin (BR) is a procaryotic ion pump. A retinal pigment that provides a link between visual pigments and BR has recently been described; the phototaxis receptor bacterial sensory rhodopsin (SR) (5, 6). SR was detected in *Halobacterium halobium* mutants that lack light-driven ion pumping (7). Like BR, SR resides in the *H. halobium* membrane, and like visual rhodopsins, SR is a sensory transducer rather than an electrogenic ion pump.

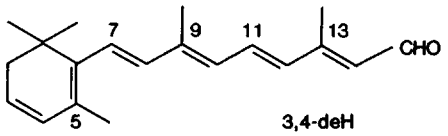
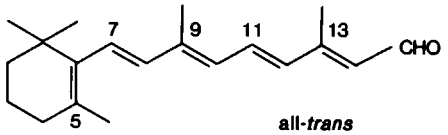
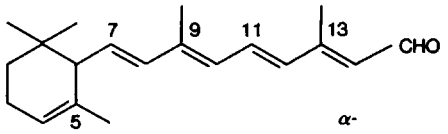
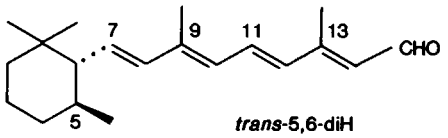
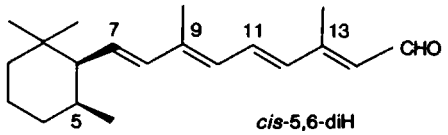
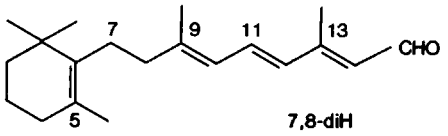
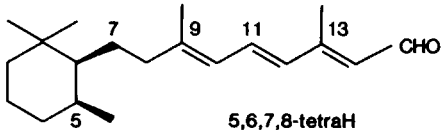
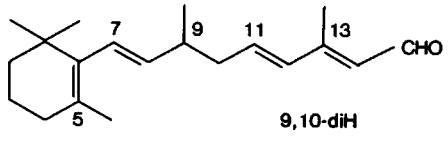
Mutants have been isolated that produce the SR-opsin, contain no other detectable retinal-binding apoproteins, and, furthermore, are blocked in retinal synthesis (7–9). We report here the use of membranes from one of the mutants (strain F1x3R) to introduce retinal analogues into the SR-opsin retinal pocket and probe retinal/protein interactions. The retinal analogues employed include the hydrogenated series (10), which have proven to be extremely useful. The dihydroretinals (see Table I) bind in vitro to give a set of data that reflects the chromophore length; analysis of their binding to bovine opsin and bacterioopsin has led to proposals of two external point-charge models dealing with the electrostatic interactions in the respective protein pockets (11, 12). Remeasurement of the set of dihydro-BR analogues, however, have shown that the maximum for 7,8-dihydro-BR is at 440 nm (Table I) rather than at 400 nm (12), and this has led to a modification of the earlier model proposed for BR (12). Dihydroretinals have also been used to induce action spectral shifts in *Chlamydomonas*, thus demonstrating that phototaxis is retinal-dependent in this organism (13). In the present study, we used F1x3R membranes to bind the dihydroretinals to SR and to compare the electronic absorption maxima with the BR series. The *cis*-5,6-dihydroretinal **5**, the dihydro-β-ionone conformation of which differs considerably from that of the *trans* analogue **4**, has been prepared for the first time and bound to the apoproteins.

MATERIALS AND METHODS

All-*trans*-retinal was purchased from Sigma Chemical Co. (St. Louis, MO) and 3,4-dehydroretinal from Eastman Kodak. Preparation of *cis*-5,6-dihydroretinal **5** was carried out as follows: β-ionone was hydrogenated >5% $Pd/CaCO_3$ (poisoned with Pb) in benzene to yield *cis*-5,6-dihydro-β-ionone, 60% yield, which was elaborated further to *cis*-

TABLE I

ABSORPTION MAXIMA IN NM, EXPERIMENTAL AND CALCULATED (UNDERLINED); THE LATTER ARE BASED ON THE MODEL OF FIG. 1. OS ARE IN CM^{-1}

	Entry	–CHO* max	–SBH⁺‡ max	SR‖ max	SR‖ OS¶	BR§ max	BR§ OS
1	 3,4-deH	396	471 458**	620 632	5,000 5,410‡‡	600 610¶	4,560 4,840‡‡
2	 all-*trans*	382	445 448	587 590	5,440 5,370	567 573	4,830 4,870
3	 α-	368	431 435	463 492	1,602 2,660	484 487	2,540 2,450
4	 *trans*-5,6-diH	370	430 435	486 492	2,684 2,660	478 487	2,340 2,450
5	 *cis*-5,6-diH	369	432 435	483 492	2,446 2,660	467 487	1,740 2,450
6	 7,8-diH	342	392 392	460 441	3,770 2,830	440 439	2,780 2,730
7	 5,6,7,8-tetraH	339	392 392	435 441	2,520 2,830	435 439	2,520 2,730
8	 9,10-diH	284	322 336			343 368	1,910‖‖ 2,590

Calculated values are underlined; the SBH^+ values were calculated with a counter-ion distance of 3.0 Å, and a ring-chain dihedral angle of 45°. For SR and BR, the parameters of the model in Fig. 1 were used.

*Absorption maxima of aldehyde in MeOH.

‡Absorption maxima of *n*-butylamine protonated Schiff base in MeOH.

‖Absorption maxima of SR in Flx3R vesicles, 1 h in the dark after binding.

§Absorption maxima of BR in bleached purple membrane, light adapted.

¶Opsin shift in cm^{-1}.

**The discrepancy in the calculated and measured absorption maxima for this compound may be due to a different conformation because of the additional double bond in the ring of 3,4-deH SBH^+.

‡‡Because of the difference between experimental and calculated maxima of 3,4-deH SBH^+ (footnote **) these calculated OS's are from the experimental value (471 nm).

‖‖Since it was found that the light-adapted 9,10-diH pigment that absorbed at 325 nm (reference 12, also present studies; OS 300 cm^{-1}) did not reversibly dark-adapt, the 343-nm value given in the table is for the dark-adapted species (unexposed to light).

5,6-diH **5** as for the *trans* series. Other dihydroretinals and 5,6,7,8-tetrahydro-retinal were prepared as previously described (10).

SR absorption spectra were determined in F1x3R vesicles prepared as previously described (7). An ethanolic solution (10 μl) of the retinal analogue (25–30 OD at 380 nm) was added to 1-ml vesicle suspension at 3.3 mg protein/ml and absorption spectra measured after 1–2 h in a Hitachi 110B spectrophotometer (Mountain View, CA) with an integrating sphere, except for analogues **3** and **6**, which required overnight incubation for maximum pigment generation (pathlength, 1 cm, 23°C, pH 6.5). Spectra from a similar procedure applied to generate SR from all-*trans*-retinal are shown in Fig. 1 of reference 8.

The BR apoprotein was prepared by NH_2OH bleaching (14). To this were added the retinal analogues (1 OD:1 OD), using an ethanolic solution (10 μl) from stock solution (100 OD at 380 nm), in the dark, overnight, at room temperature in distilled water; the pigment suspension was irradiated for 15 min at room temperature with orange light from a 1,000 W lamp equipped with a Corning 3-69 glass filter and heat filters (Corning Glass Works, Corning Medical and Scientific, Corning, NY). π-Electron calculations of absorption maxima were carried out as described previously (11, 12, 15, 16, 17).

RESULTS

The absorption maxima of the SR and BR chromophores formed from the retinal analogues are considerably red-shifted relative to the corresponding protonated Schiff bases (SBH^+) (the expected linkage in rhodopsins) formed with *n*-butylamine (Table I). This bathochromic shift, expressed in cm^{-1}, has been defined as the "opsin shift" (12) and represents the environmental effect of the protein binding site on the absorption maxima of the pigment. Table I lists the absorption maxima for the analogues, the SBH^+ forms, the SR and BR pigments, and the opsin shifts, both experimental and calculated.

Four of the analogues in Table I, 3,4-dehydroretinal (3,4-deH) **1**, *trans*-retinal **2**, *trans*-5,6-dihydroretinal (*trans*-5,6-diH) **4**, and 7,8-dihydro-retinal (7,8-diH) **6**, provide a series of all-*trans* retinals having incrementally diminishing π-orbital systems to interact with the opsin-binding domain. An important result in Table I is that SR and BR show a similar pattern of opsin-shift values, SR giving consistently greater opsin shift values than BR for each of the four retinals. In both cases the largest opsin shift occurs with the natural retinal **2** of the organism. Both show a 33-nm increase in λ_{max} and slight decrease in opsin shift with 3,4-deH **1** compared with *trans*-retinal **2**. The opsin shifts of the *trans*-5,6-diH-**4**–derived analogues are ~50% of the *trans* retinal values for both SR and BR, and the shifts for 7,8-diH **6** are greater than those of the *trans*-5,6-diH **4** pigments.

Less close correspondence is observed between the SR and BR analogues formed from *cis*-5,6-diH **5**, which has a conformation differing considerably from that of *trans*-5,6-diH **4**; a difference is also seen between the SR and BR analogues derived from α-retinal **3**. Additionally, in SR there is a considerable drop in opsin shift from 7,8-diH **6** to 5,6,7,8-tetrahydroretinal **7** analogues. These differences are discussed below in terms of differing steric requirements of the SR and BR apoproteins. Independent of the particular model used to describe these effects, the results show that the overall structures of the retinal binding sites in SR and BR are basically similar.

DISCUSSION

The opsin shifts of the dihydroretinal series have been interpreted in terms of through space electrostatic interactions between charged amino acids on the protein and the retinal (11, 12). Two interactions were used to explain the previously reported data for BR: (*a*) a closely associated counter-ion to the protonated Schiff base of the retinal and (*b*) a negatively charged group positioned near C-5 in BR, and near C-12/C-14 in bovine rhodopsin. In the proposed models, the nitrogen counterion distance was fixed so as to reproduce the absorption maxima of model SBH^+ compounds in solution (11, 12, 17). Thus, the opsin shift was attributable solely to the second negative charge. It is of interest to reconsider the spectroscopic determinants in BR in light of the data of Table I.

It was found that 7,8-dihydroretinal **b** led to a considerably larger opsin shift in BR (2,780 cm^{-1}, Table I) than previously reported (maximum at 400 nm, OS 1,000 cm^{-1} (12, 18).[1] Since the red shift of the 7,8-dihydro pigment cannot be due to a charge near the ring, the data imply that the nitrogen/counter-ion interaction is weaker in native BR and analogues than in the corresponding protonated Schiff bases with butylamine. A weaker nitrogen/counter-ion interaction, which would lead to increased delocalization of the positive charge and increased red shift, could result, for example, from a greater nitrogen/counter-ion distance, a possibility first suggested by Blatz and Mohler (19); it is also consistent with solid state ^{15}N chemical shift studies (20). The nearly constant opsin shifts of 2,500 cm^{-1} for retinal analogues that lack a β-ionone ring have also suggested that interactions near the Schiff base play an important spectroscopic role (21). A second factor that should be considered in deriving a model is the conformation of the chromophore around the 6-7 single bond (22, 23) (see discussion on planarity below).

Further support for the existence of spectroscopically important interactions in the vicinity of the β-ionone ring comes from an analysis of the opsin shifts reported in Table I. Both 7,8-diH BR and SR exhibit larger opsin shifts than the corresponding 5,6-dihydro pigments, but both sets of opsin shifts are smaller than those of the native chromophore. This trend points to the presence of a highly specific set of interactions in the vicinity of the ring, which reduce the opsin shift of 5,6 pigments while increasing the shift of the full chromophore relative to the opsin shift of the 7,8-dihydro pigment. A single negative charge, as origi-

[1]This trend was first noticed when the absorption maxima of dihydro-BR's were remeasured in conjunction with comparisons of the proton translocation efficiencies (see reference 18). We suspect that the 400-nm reported for 7,8-diH-BR in reference 12 (instead of the present 440-nm value) was due to inadequate preparation of the chromophore or pigment.

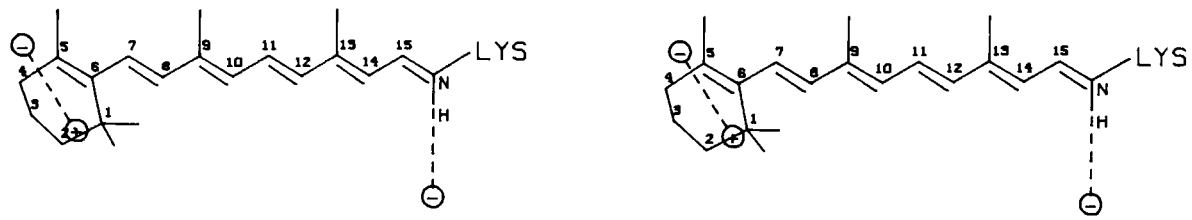


FIGURE 1 Stereo images for a point charge model for BR and SR. The Schiff base counter-ion is 3.9 and 4.0 Å from the Schiff base nitrogen in BR and SR, respectively. The ring negative charge is 3.0 Å above C-5. The angle between the ring negative charge, C-5 and C-6 is 95°. The positive charge by the ring is 3.0 Å from the negative charge. The angle between the positive charge, the negative charge and C-5 is 76° in BR and 78° in SR. The dihedral angle formed by the ring positive charge, negative charge, C-5 and C-7 is 60° for both pigments.

nally postulated (12), is clearly inadequate since this could not account for the decreased opsin shift of the 5,6-dihydro pigments. This can be done by assuming the existence of a positive charge that is located so that it depresses the opsin shift of the 5,6-dihydro chromophore. Such a charge was alluded to as forming a salt bridge with the negative charge near C-5 (12), but based on the available opsin shifts it was assumed that it was well removed from the β-ionone ring. The new set of data (Table I) now require that the original model be modified so as to include the effects of both members of the ion-pair. Presence of an ion pair is also consistent with NMR data (23).

The new model (Fig. 1) was chosen so as to produce a satisfactory fit of calculated values to experimentally observed opsin shifts (Table I). The general features of the model were derived from the following considerations: An increased nitrogen-counter-ion distance of 3.9–4.0 Å (as compared to the ~3-Å distance of the protonated Schiff bases with *n*-butylamine [17]) was chosen to account approximately for the opsin shifts of the 9,10- and 7,8-dihydro-pigments. The large opsin shift of the full chromophore is accounted for, in part, by assuming a planar s-*trans* ring-chain conformation, as has recently been concluded based on NMR evidence (23). This planarity factor applies only to the native chromophore, since the ring is not part of the system in any of the dihydro pigments. However, planarity and an increased counter-ion distance do not account for the full opsin shift of the native chromophore. Even when these two factors are considered, the calculated opsin shift is still ~1,000 cm^{-1} short of the experimental value. The full opsin shift of BR and SR are reproduced only if a negative charge near C-5 is included.

It appears then that the absorption maxima of BR and SR are due to a combination of factors, including a weakened counter-ion interaction, a planar ring-chain conformation, and an ion-pair near the β-ionone ring. The negative end of the ion-pair must be near C-5, while the positive end is nearer to the 7,8-double bond. The specific geometry shown in Fig. 1 places the positive charge above the β-ionone ring near C-6 and C-1, ~3.7Å from C-7. Other locations above the polyene chain near C-7 are also possible, but these lead to a configuration of the ion pair that results in unacceptably large opsin shifts for 3,4-dehydro retinal. Thus, Fig. 1 accounts for the entire set of opsin shifts. It should be emphasized, however, that the model is only an approximate representation of the true protein-chromophore interactions. It is obviously an over-simplification to treat a carboxylate anion, the most likely source of the negative charge, as a point charge.

In addition to the electrostatic factors, the data clearly indicate, in agreement with the recent study of Sheves et al. (24), that stereochemistry in the ring end of the chromophore plays an important spectroscopic role. For example, the substantially different opsin shifts of *trans*- and *cis*-5,6-dihydroretinals **4** and **5** in BR or those of 7,8-dihydro- (which is considerably higher than the calculated value) and 5,6,7,8-tetrahydroretinals **6** and **7** in SR indicate that ring conformation is an important factor, even when the ring is not part of the π-conjugated system. This would be expected if steric interaction with the protein determined the detailed orientation of charged or polar amino acids on the protein with respect to the chromophore.

Note that despite the similarities, the retinal binding pocket of the SR apoprotein appears to have a somewhat different shape from that of the BR apoprotein, at least in the vicinity of the β-ionone ring. For example a large difference between the *trans*- and *cis*-5,6-dihydro-opsin shifts occurs in the BR but not in the SR pigment.

The function of SR and BR are different, the former being responsible for photosensory signaling, while the latter is responsible for light-driven proton translocation. Although the two proteins differ in their steric requirements in a subtle manner, the overall chromophore/protein interaction depicted in Fig. 1 applies to both. Thus the inherent electrostatic and steric properties of the retinal binding site structures have been conserved in the SR and BR apoproteins despite their different functions.

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