CHROMOPHORE/PROTEIN AND CHROMOPHORE/ANION INTERACTIONS IN HALORHODOPSIN

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ABSTRACT Halorhodopsin (HR), the light-driven chloride transport pigment of *Halobacterium halobium*, was bleached and reconstituted with retinal analogues with the pi electron system interrupted at different locations (dihydroretinals). The absorption maxima of the artificial pigments formed with the dihydroretinals are found to be very similar to those of the corresponding pigments formed by reconstitution of bacteriorhodopsin (BR) and sensory rhodopsin (SR). This strongly suggests that the distribution of charges around the retinal is similar in all three bacterial rhodopsins. Comparison of the primary, and proposed secondary, structures for HR and BR reveal conserved asparagine (asp) and ariginine (arg) residues, which are likely candidates for the ionizable amino acids that interact with the retinal. In a second set of experiments absorption shifts due to the binding of anions to Sites I and II in HR, reconstituted with different retinal analogues, were used to estimate the locations of these binding sites relative to the retinal. Site I is localized near the Schiff base, and Site II near the ionone ring. On the basis of these results a structural model for HR is proposed, which accounts for the spectroscopic properties of HR in terms of the three buried arg residues and two of the buried asp residues in the protein.

INTRODUCTION

Visual and bacterial rhodopsins contain a retinal chromophore, bound via a protonated Schiff-base to the epsilon-amino group of a lysine. The absorption spectrum of the chromophore appears to be determined largely through electrostatic interactions between the chromophore and positively and negatively charged amino acids located in or near the retinal binding site. Information as to the position of these charges has been obtained from analysis of the absorption spectra of artificial pigments formed from retinal analogues (dihydro retinals), in which the pi electron system is interrupted at different locations (Spudich et al., 1986; Lugtenburg et al., 1986), and from ^{13}C nuclear magnetic resonance chemical shifts in bacteriorhodopsin (BR)[1] isotopically labeled in the retinal (Harbison et al., 1985).

Here, dihydro retinals are used to characterize the spectroscopic determinants in the light-driven chloride pump of halobacteria, halorhodopsin (HR).[1] A number of recent studies have provided fairly detailed models of the chromophore–protein interactions in the other two retinal pigments in the cytoplasmic membrane of the halobacteria, bacteriorhodopsin (BR) and sensory rhodopsin (SR). The magnitude of protein–chromophore interactions is usually described in terms of "opsin shifts," which are shifts in the absorption maximum of the protein-bound chromophore relative to that observed for model compounds in organic solvents. In BR and SR about half the opsin shift appears to originate from a Schiff-base counterion interaction that is weaker than in the model compounds, while the other half is due to interactions in the vicinity of the ionone ring (Spudich et al., 1986; Lugtenburg et al., 1986). The latter involves the effects of both a positively and a negatively charged amino acid residue, probably an ion-pair, as well as steric effects which lead to a more planar ring-chain conformation than is found in model compounds. The remarkable similarity of the opsin shifts for BR and SR (Spudich et al., 1986) implies that chromophore–protein interactions in the retinal binding site are well-conserved in the two pigments, despite their widely different functions. It was therefore of considerable interest to determine whether the same interactions are present in HR as well.

Besides the large opsin shift, smaller shifts in the absorption maximum (in both the red and blue directions) have been observed upon addition of various anions to HR (Ogurusu et al., 1982; Steiner et al., 1984; Schobert et al.,

[1] *Abbreviations used in this paper:* arg-xxx and arg-(xxx), numbered arginine residues in HR and BR, respectively, as are other residues; BR, bacteriorhodopsin; HR, halorhodopsin, octylglucoside, *n*-octyl-beta-D-glucopyranoside; SR, sensory rhodopsin.

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1986; Lanyi, 1986; Schobert and Lanyi, 1986). These shifts and their dependencies on the concentration of the anions have suggested the existence of anion binding sites (Schobert et al., 1986): Site I, which is relatively nonspecific, and Site II, which binds only chloride and bromide, the anions which are transported by HR. Anions binding to Site I give a 5–10 nm blue shift, while those binding to Site II produce a similar shift but in the opposite direction. Chloride and bromide, which bind to both of these sites, give little or no spectroscopic change, apparently because the shifts cancel each other. Both the specificity and the binding affinity of Site II suggests that it participates in the binding of the chloride ion during its translocation. A role also for Site I in the transport cannot be ruled out. Based on the interpretation of the anion-dependent shifts as being due to electrostatic interaction of the negative charges with the retinal, Site I was tentatively localized near the Schiff-base and Site II near the ionone ring (Lanyi, 1986). This model is supported by the fact that the pK_a of the Schiff-base is raised by as much as 2 units upon occupancy of Site I, but remains relatively unaffected by additional occupancy of Site II (Schobert et al., 1986; Schobert and Lanyi, 1986).

We report here the results of reconstituting HR with dihydro retinals, in experiments similar in design and intent to those with BR and SR (Spudich et al., 1986; Lugtenburg et al., 1986). Such reconstitutions have been very difficult to do with HR, particularly when using retinal analogues. The data obtained show great similarity to what was found for the other two halobacterial rhodopsins, indicating that the spectroscopic determinants in the retinal binding sites of these three pigments must be virtually identical. The data also confirm the previously proposed locations for Sites I and II relative to the retinal.

Since the primary structure of HR has recently been determined by Blanck and Oesterhelt (1987), it was possible to seek common features in the BR and HR sequences that might account for their similar spectroscopic properties. Analysis of the structural models proposed (Blanck and Oesterhelt, 1987; and in this paper) suggests that the counterion to the Schiff base is asp-238 (corresponding to asp-[212] in BR), and the ion-pair near the ionone ring may be the conserved aspartate (asp)-141 and arginine (arg)-200 in HR (corresponding to asp-[115] and arg-[174] in BR). Specific arg residues which constitute Sites I and II in HR are also suggested.

MATERIALS AND METHODS

All-*trans* retinal was purchased from Fluka, and 13-*cis* retinal from Eastman Kodak Co., Rochester, NY. Cardiolipin (bovine heart) was from Sigma Chemical Co., St. Louis, MO. All-trans dihydro-retinals were prepared as previously described (Arnaboldi et al., 1979). 3,4-Dehydroretinal was prepared from 3,4-dehydro-beta-ionone, which was synthesized as described in Surmatis and Thommen (1967). 3,4-Dehydro-beta-ionone was converted to all-*trans* and 9-*cis*-C_{15}-aldehyde through conventional Emmons reaction with ethyl phosphonoacetate (Wadsworth et al., 1961), dibal reduction, MnO_2 oxidation and flash chromatographic separation of isomers. A second series of the same C_5 elongation process, carried out with the all-*trans*-C15-aldehyde and a mixture of *cis/trans*-ethyl-phosphonosencioate (Olive et al., 1969), gave the all-*trans* and 13-*cis* isomers of 3,4-dihydroretinal. 13-14 Five-membered ring-locked retinals were synthesized as previously reported (Fang et al., 1983).

HR was prepared by chromatography of *Halobacterium halobium* OD2W membrane proteins on Phenylsepharose in the presence of cholate and octylglucoside. The purified pigment was stored at 4°C in the dark in 4 M NaCl, 25 mM Tris.Cl pH 7.2, 0.5% octylglucoside; its concentration was 15–30 nmol/ml, as estimated from an extinction coefficient of 50,000 $M^{-1} \cdot cm^{-1}$ at 580 nm (Lanyi and Weber, 1980). Before bleaching a lipoprotein complex was produced, in the following way. A few milligrams of cardiolipin were dried under nitrogen, followed by repeated redissolution in a few drops of diethyl ether and re-drying. The lipid was dispersed in a small volume of water by brief sonication and addition of octylglucoside to 2% final concentration. The lipid dispersion (4–5 mg/ml) was added to HR in an amount to give a protein/lipid ratio of 3 (wt/wt), and the mixture was dialyzed against 3 × 100 vol of 4 M NaCl, 25 mM Tris.Cl, pH 6.0. Bleaching was at 10°C, in the presence of 0.2 M hydroxylamine, freshly prepared and adjusted to pH 6, under intense illumination through a 530-nm long-pass filter. The pigment was ~85% bleached after 8 h of illumination, as determined from the disappearance of absorption at 580 nm. The sample was then dialyzed against 3 × 100 vol of 4 M NaCl buffer, as above, and either used for reconstitution in this buffer directly, or the buffer was first replaced with 0.4 M Na_2SO_4, 25 mM Tris.Cl, pH 6.0 containing either 0.1 M NaCl, 0.1 M $NaNO_3$, or neither of these as indicated in the text, by repeated centrifugation at 12,000 *g* for 10 min and resuspension. Changing the buffer after the reconstitutions was not feasible. As before (Lanyi, 1986), reconstitutions were followed as difference spectra with a UV-250 (double monochromator) (Shimadzu Scientific Instruments Inc., Columbia, MD) spectrophotometer, after adding retinal or retinal analogue in ethanol solution to the sample in the measuring cuvette, and an equal volume of ethanol (not exceeding 1% of the total) to the reference cuvette. The spectra were recorded over time periods ranging from 1 to 2 h to 2 d with different retinal analogues. Reconstitution yields also varied greatly with the retinal analogue used, from a few percent to ~50%. Some retinal analogues gave little or no reconstitution in some of the buffers.

The absorption maximum of 9-*cis* HR ("iso-halorhodopsin"), produced during lengthy illumination with red light (Zimányi and Lanyi, 1987), was determined by computer-aided graphic resolution of composite spectra of samples illuminated for various lengths of time through different color filters, as described elsewhere. Because 9-*cis* HR is produced preferentially when the protein is in a phosphatidylcholine/phosphatidylethanolamine bilayer, and therefore the experiments were done in the presence of these lipids, the spectra obtained for this HR analogue are not necessarily comparable to the reconstitution spectra, where the lipid was cardiolipin.

RESULTS

The bleaching and reconstitution of HR is far more difficult than the procedures that are effective for other rhodopsins. Cell envelope–bound HR bleaches very slowly (Lanyi and Weber, 1980), and the SR always present in these membranes (Bogomolni and Spudich, 1982) interferes with the measurements of the reconstituted HR spectra. Purified, detergent-solubilized HR is rapidly bleached in the presence of hydroxylamine, but the product does not reconstitute with added retinal (results not shown). We have been unable to reproduce a published reconstitution procedure (Bogomolni et al., 1984) for

detergent-free HR, and an alternative method we had developed (Lanyi, 1986) has worked well for retinal but very poorly for the dihydro-retinal analogues.

We find that reconstitution is reasonably effective when a lipoprotein complex is formed from purified HR with the detergent removed, as described in the Materials and Methods section, but the success of the procedure depends both on the kind of lipid used, i.e., cardiolipin, and on high protein/lipid ratios. Even so, we found that some retinal analogues which reconstitute in other rhodopsin systems, such as 9,10-dihydro retinal and 13-*cis*-locked retinal, did not reconstitute at all, and several of the dihydro series reconstituted well only in 4 M NaCl, the physiological salt concentration for the halobacteria.

Opsin shifts were determined by reconstituting in 4 M NaCl, where all reconstitutions were the most extensive. Fig. 1 shows some representative spectra, and Table I gives the absorption maxima (in nanometers) and opsin shifts, expressed as frequency differences (in cm^{-1}), for HR, and for BR and SR from the previous study (Spudich et al., 1986). It is apparent from Table I that the opsin shifts for the three bacterial rhodopsins are very similar, although all the absorption maxima, except for the 9-*cis* chromophore, are somewhat red-shifted for HR relative to BR. Some of the data in Table I are plotted in Fig. 2, where the opsin shift for HR is seen to correlate well with the opsin shift for BR. Least-squares fit of the points gave a line with the equation, $OS_{HR} = 0.99\ OS_{BR} + 380$, indicating that the larger opsin shift for HR is accounted for by a constant increment of 380 cm^{-1} relative to the opsin shift of BR, regardless of the retinal analogue used.

The shifts caused by anion binding to Sites I and II were calculated from absorption maxima determined from reconstitutions in sulfate, sulfate + nitrate, and sulfate + chloride, as described in the Materials and Methods section, with the rationale (Schobert et al., 1986; Lanyi, 1986) that in sulfate both sites are unoccupied, in sulfate + nitrate only Site I is occupied, and in sulfate + chloride both sites are occupied. Thus, the Site I shift is obtained from the difference between the absorption maxima in sulfate + nitrate and sulfate, and the Site II shift from the difference between sulfate + chloride and sulfate + nitrate.

Table II shows the results, expressed as absorption maxima (in nanometers) and frequency shifts in analogy with the opsin shifts (in cm^{-1}). The data in Table II

TABLE I
ABSORPTION MAXIMA (IN NM) AND OPSIN SHIFTS (OS IN CM^{-1}) FOR DIFFERENT RETINAL ANALOGUES OF HR, BR, AND SR

Retinal analogue	HR*		BR‡		SR‡	
	Max	OS	Max	OS	Max	OS
3,4 Dehydro (all-trans)§	609	4,810	600	4,560	620	5,000
All-*trans*§	578	5,170	568	4,840	587	5,440
13-*Trans* (locked)§	594	4,670	576	4,140		
13-*Cis*§	568	4,870	548	4,220		
9-*Cis*	481‖	1,940	491¶	2,360		
5,6 (*Trans*) dihydro	482	2,510	478	2,340	486	2,680
5,6 (*Cis*) dihydro	474	2,050	467	1,740	483	2,440
7,8 Dihydro	453	3,440	440	2,780	460	3,770

*Data from reconstitutions in this study, in 4 M NaCl, 25 mM Tris.Cl pH 6.0.
‡Data from (Spudich et al., 1986) and unpublished experiments.
§Reconstitution complete within a few hours.
‖From iso-halorhodopsin spectra.
¶Personal communication with T. G. Ebrey.

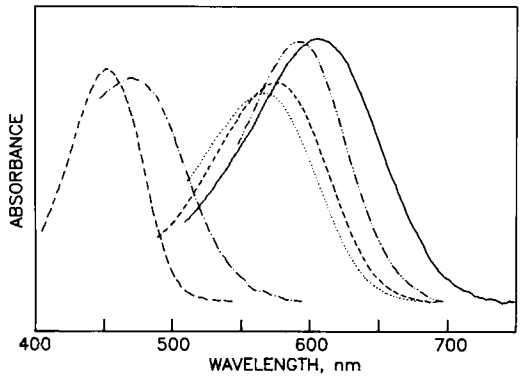

FIGURE 1 Spectra of bleached halorhodopsin reconstituted with various retinal isomers and analogues. The spectra were obtained as described under Materials and Methods, as difference spectra between two samples with the retinal isomer or analogue added to one and ethanol added to another. Symbols (the absorption scale is indicated in parentheses): (—) 3,4 dehydro retinal (0.12); (- · · · · · · · ·) 13-*trans* (locked) retinal 0.20; (- - - - - -) all-*trans* retinal (0.28); (· · · · · · · · · ·) 13-*cis* retinal (0.16); (- · · · -) 5,6 (*cis*) dihydro retinal (0.048); (– – – –) 7,8 dihydro retinal (0.096).

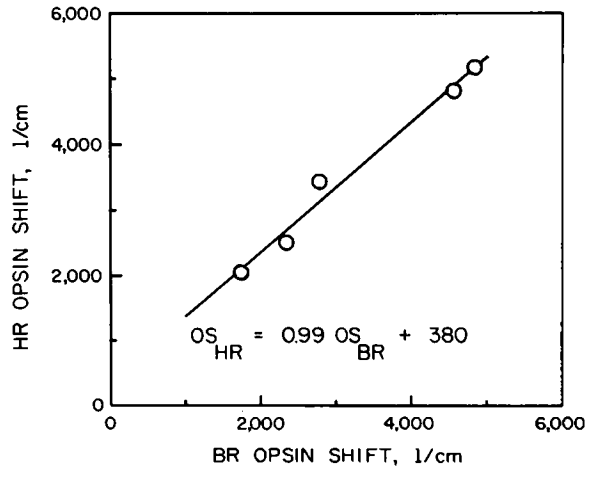

FIGURE 2 Correlation of the opsin shifts of HR and BR, reconstituted with various retinal analogues. Data from Table I for the 13-*trans* HR chromophores and from (Spudich et al., 1986) for BR. Line and equation in insert represent least-square fit. OS_{BR} and OS_{HR} are opsin shifts for BR and HR, respectively.

TABLE II
ABSORPTION MAXIMA (IN NM) AND ANION-BINDING SITE ASSOCIATED SHIFTS (IN CM^{-1}) FOR DIFFERENT RETINAL ANALOGUES OF HALORHODOPSIN

Retinal analogue	Maximum*			Shift for site	
	Nitrate	Chloride	None	I	II
3,4 Dehydro	607	612	614	−190	130
All-*trans*	566	578	573	−220	340
13-*Cis*	564	571	573	−280	220
9-*Cis*‡	480	480	485	−210	0
7,8-Dihydro	450	452	§	—	100

*From reconstitutions in 0.4 M Na_2SO_4, 25 mM Tris.Cl pH 6.0, containing either 0.1 M NaCl, 0.1 M $NaNO_3$, or neither of these, as indicated.

‡From iso-halorhodopsin, under the conditions described in *, and as in Table I.

§Insufficient extent of reconstitution with this analogue under these conditions, similarly to some of the other dihydro analogues in Table I, which gave unsatisfactory reconstitution except in the buffer containing 4 M NaCl.

indicate that the Site I shift is negative and is only slightly or not at all changed with 9-*cis* and 3,4 dehydroretinal, which involve alterations (relative to all *trans*-retinal) remote from the Schiff base. The effect of isomerization around the 13-14 double bond is somewhat larger. These results suggest that the site is located near the Schiff base, as proposed previously (Lanyi, 1986). The data in Table II indicate also that the Site II shift is positive, and is diminished by all alterations of the retinal. The decrease of the Site II shift is smallest when the isomerization is around the 13-14 double bond, and most pronounced when the isomerization is around the 9-10 double bond or the pi system is interrupted at carbon 7. Interestingly, desaturation of the 3-4 single bond also decreases the Site II shift. These results suggest that Site II is located close to the ionone ring, as we have proposed previously (Lanyi, 1986). It should be emphasized that the anion dependent shifts are quite small, and each alone could not be taken as conclusive in these arguments. However, the data together do present a quite consistent pattern, and it is this that justifies the conclusions made.

DISCUSSION

It is clear from the data presented in Table I and Fig. 2 that the opsin shifts of the dihydro-retinal pigments formed from HR closely parallel those of BR and SR. This result strongly suggests that the spectroscopic determinants in the three pigments are essentially identical: the large opsin shifts for 7, 8 dihydro-retinal are consistent with weak counterion Schiff-base interaction (Harbison et al., 1983), while the reduced shifts for the 5,6 dihydro-retinals and the large opsin shifts for the full chromophore are indicative of an ion-pair near the ionone ring (Spudich et al., 1986). Thus, it seems very likely that a conserved ion-pair will be apparent in the amino acid sequences of the three pigments, which would appear to have a common evolutionary origin.

Based on sequence comparisons with BR and hydropathy profiles, Blanck and Oesterhelt (1987) have proposed a model for the folding of the HR sequence in the bilayer, which is quite similar to that for BR. The orientation of the putative seven alpha-helices of HR in the membrane is the same as in BR, i.e., the COOH terminus is on the cytoplasmic side (Schobert, B., J. K. Lanyi, and D. Oesterhelt, manuscript in preparation). The basic and acidic residues in HR and BR in this model (modified somewhat in helix G to correspond to the structure proposed for BR by Engelman et al., 1980, 1986) are shown in Fig. 3. Conserved residues are depicted in capital letters. According to this model, HR contains fewer buried charged residues than BR, and many of them, including all of the

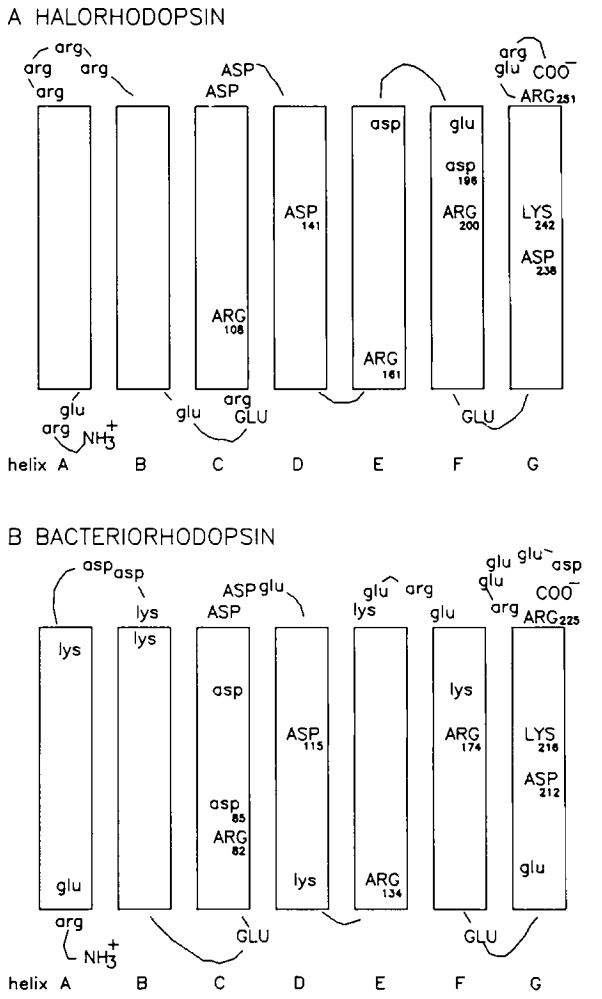

FIGURE 3 Secondary structures proposed for HR (*A*) and BR (*B*) with only acidic and basic residues shown. Residues with capitalized letters are conserved in the sequence. The HR structure is as proposed by Blanck and Oesterhelt (1987), with a shift in helix G to bring the sequence in line with the BR structure of Engelman et al. (1980, 1986). Numbering of the residues in HR is after Blanck and Oesterhelt (1987), which includes an initial sequence in the gene not found in the protein. The BR structure is from (Engelman et al., 1980).

arg residues, are conserved in the two proteins. It is evident from Fig. 3, however, that only two of the buried acidic residues are conserved: asp-238 (212) and asp-141 (115). It has been stated many times before that in BR the asp residue on helix G, one helical turn away from the lysine bearing the retinal, is the most likely candidate for the Schiff base counterion. Thus, since asp-238 (212) on helix G is probably the Schiff-base counterion (Blanck and Oesterhelt, 1987), asp-141 (115) on helix D appears to be the best candidate for the negative charge near the beta-ionone ring. The fact that in both sequences this highly polar residue is surrounded by hydrophobic residues (which would be expected to interact favorably with the ring carbon atoms) supports this assignment. With regard to the positive charges, four or possibly five basic amino acids are predicted from the model of Blanck and Oesterhelt to be within the lipid bilayer: lys-242 (216), which binds the retinal, as well as arg-108 (82), arg-161 (134), arg-200 (174), and arg-251 (225). If the BR structure proposed by Engelman et al. (1980) is adopted, as done in Fig. 3, arg-251 (225) is not buried but located at the aqueous interface of helix G. The results of Ariki et al. (1986), which indicate that only three of the arg residues in HR do not react with phenylglyoxal, are consistent with the existence of three buried arg residues. Of the three (or four) buried arg residues, only arg-200 is predicted by Blanck and Oesterhelt to be located at the same depth within the bilayer as asp-141 and, on this basis, would appear to be the most likely candidate for the positively charged residue forming an ion-pair with asp-141 near the beta-ionone ring. An independent analysis of the BR sequence leads to a similar conclusion. For example, Engelman et al. (1980) placed asp-(115) and arg-(174) at approximately the same depth in BR. In a more recent study, using a newly developed hydropathy algorithm, Engelman et al. (1986) predict that asp-(115) in BR lies in the interior of the bilayer but closer to the cytoplasmic than to the extracellular side of the membrane. Arg-(174) is predicted to be at the cytoplasmic surface but the authors point out that the F helix appears to begin a few residues earlier than they predict. This would place arg-(174) inside the bilayer in a position to interact with asp-(115). No other arg residue in BR is predicted in any published algorithm to be close enough to asp-(115) to form a salt bridge. Thus arg-200 in HR and arg-(174) in BR are strongly indicated as the basic residues that interact with the retinal chromophore in the vicinity of the beta-ionone ring.

Given this analysis, it is of interest to consider the source of the small red shift for HR relative to BR. If arg-200 is the positive charge near the ionone ring in HR, it would be expected to interact with asp-196 (possibly forming a bifurcated ion-pair with asp-196 and asp-141). Since asp-196 corresponds to lys-(170) in BR, the extra negative charge near the ring in HR would be expected to produce the observed red shift. While this explanation appears entirely plausible, Raman evidence suggests a different source for the red-shift: weaker Schiff base–counter ion interaction rather than modified interactions near the ring. The evidence is that HR exhibits a C=N stretching frequency ~7 cm^{-1} lower than that of BR and a much smaller shift of this frequency in D_2O (Smith et al., 1984). Since both of these observations are consistent with weaker counterion interaction or weaker hydrogen bonding (Aton et al, 1980; Sheves et al., 1987; Gilson H., and B. Honig, manuscript in preparation), a spectral perturbation near the Schiff base appears to be quite likely. If the source of the small red-shift in HR is near the Schiff base, one is faced with the apparently contradictory observations that the sequences of BR and HR are almost identical in the region around the active site lysine, despite their spectroscopic differences. In particular, asp-238 and asp-(212), the putative counterions, are both one turn of a helix from the lysine on helix G in the two pigments, and might be expected to interact identically in the two cases. However, this interaction would be weakened in HR if the asp were to interact with another residue in this protein. The evidence that chloride binding Site I is located near the Schiff base suggests that an additional arg should be located in this vicinity in HR. This group, if it interacts with asp-238, could disrupt its interaction with the Schiff base, thus producing the observed red shift and changes in the Raman spectrum. An attractive candidate for this residue is arg-108 which, based on the folding model of Blanck and Oesterhelt, could easily extend to the vicinity of the Schiff base. Consistent with this suggestion is the fact that in BR there is an asp on helix C one turn of a helix from the corresponding arg (arg-82). Thus in BR arg-(82)

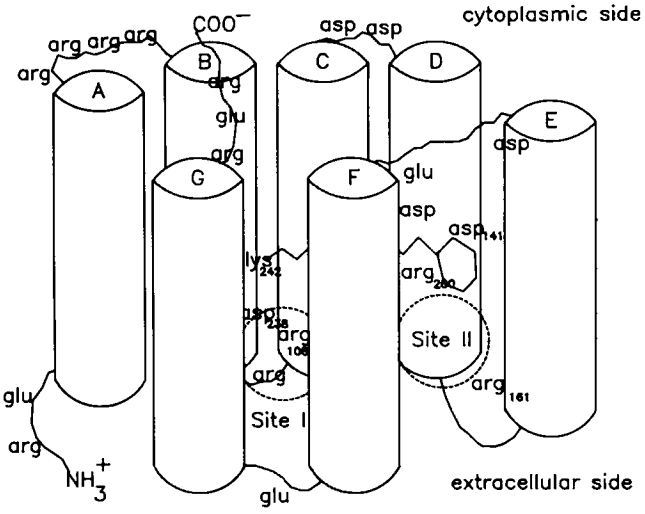

FIGURE 4 Proposed structural model for HR. For helical designation and location of residues, see Fig. 3 *A*. The model is based on the assignment of arg-200 (on helix F) and asp-141 (on helix D) as the two charged residues which interact with the ionone ring, which brings the helices into much the same positions as in one of the proposed models for BR (Engelman et al., 1980). Anion binding Sites I and II are localized where indicated with circles, as explained in the text.

would be expected to form a local ion pair with asp-(85) while in HR arg-108 would be free to assume a different conformation.

The proximity of specific residues suggested by this analysis makes it possible to suggest a crude model for HR and for the position of the retinal relative to its seven helices (Fig. 4). The beta-ionone ring is predicted to be close to asp-141 on helix D and to arg-200 extending from helix F. Note that the sequential organization of the helices is assumed to be that proposed in the original model of Engelman et al. (1980) for BR. Other helical assignments have been proposed also (Seiff et al., 1985, 1986). However, the model of Engelman et al. (1980) places helices D and F in close proximity to one another and to a location in the neutron density map believed to be near the ionone ring (Seiff et al., 1985). Thus, the results of this work are consistent with the original model of Engelman et al. although it is likely that some other models would also predict ion-pairs close to the ionone ring.

In the HR model shown in Fig. 4 all three of the buried arginines appear to be fairly close to the retinal. Since Ariki et al. (1986) found that chemical modification of the accessible arg residues did not significantly alter the two anion binding sites, the residues responsible for anion binding should be sought among these buried arginines. As discussed above, Site I should be close to the Schiff base (although not in immediate contact; Schobert and Lanyi, 1986; Maeda et al., 1985). An appropriate location, at arg-108, is sketched into the figure. Binding of an anion to this site would produce a blue shift and decrease in pK, as is observed. We have carried out pi electron calculations (see e.g., Kakitani et al., 1985) which suggest that a negative charge located ~8 Å from the Schiff base and interacting with an effective dielectric constant of 10 (reflecting screening by both the low dielectric protein and the high dielectric solvent [see e.g., Honig et al., 1986]) would reproduce the observed blue shift induced by anion binding to Site I. Such an interaction would be predicted to induce a pK shift of ~3 units, on the order of what is observed for chloride and bromide. However, this simple model involving direct through-space interaction between the anion and the Schiff base leaves unexplained the remarkable relationship between the binding energies of different anions and the pK shifts they induce at the Schiff base (Schobert and Lanyi, 1986).

Previous evidence (Lanyi, 1986) as well as the results of this work suggest that Site II is located near the beta-ionone ring. Arg-200, which we localize near the ring, might participate in that site. The other residue, which might act in concert with arg-200 as suggested by the model of Fig. 3, is arg-161. All other arg residues are located outside the bilayer, and cannot be considered eligible for interaction with chloride near the retinal. The tentative location of Site II, consistent with these arguments, is also sketched into the model. Significantly, the assigned locations for the two anion binding sites both fall on the extracellular side, the side to which they have been found to have rapid diffusional access (Lanyi et al., 1983; Schobert et al., 1983; Bogomolni et al., 1984), and from which chloride is taken up during transport. Given the fact that all arg residues in HR which we propose to bind chloride are conserved in BR (Fig. 2), the question naturally arises whether BR might not be transporting hydroxyl ions by a similar mechanism, rather than protons. In this connection we wish to point out that the binding constant for chloride by the two sites in HR is $\sim 10^{-2}$ M (Schobert and Lanyi, 1982; Steiner et al., 1984), and there is no reason to believe that a binding mechanism for OH^-, based on the arg residues in BR, would have much higher affinity for this anion. Thus, unless other buried residues in BR (Fig. 2 *B*) also participate in the OH^- binding, hydroxyl transport at the low OH^- concentration near neutral pH seems extremely unlikely.

The structural model in Fig. 4 suggests possible transport schemes, involving binding of the chloride by Site II on the extracellular side, its transfer inside the protein, and its release on the cytoplasmic side, perhaps away from the acidic region above helices C, D, and F but through the positively charged domain above helices A, B, and G. Such models will be described and evaluated elsewhere.

Finally, it is worth noting that HR lacks free lysines, and thus arginines rather than lysines will participate in the anion transport process. This can be rationalized as follows. The ionization state of lysines in the low dielectric environment of the membrane, even if they participate in an ion-pair, will depend very sensitively on their immediate hydrogen-bonding environment (Honig et al., 1984). In contrast, the high pK of arginines and the fact that their charge is distributed over a larger effective radius than in a lysine, facilitates the ionization of arginines, even in a low dielectric medium.

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