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Studies with retinal pigments: modified point charge model for bacteriorhodopsin and difference FTIR (Fourier transform infrared) studies

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Abstract - Incorporation of dihydroretinals into the sensory rhodopsin SR and bacteriorhodopsin bR has led to a modified version of the external point-charge model for bR proposed in 1979. Difference Fourier transform infrared studies of native bR and bR analogs cultured on media containing isotopes of amino acids have shown some of the amino acids which undergo environmental changes or deprotonation/protonation during the bR photocycle leading to proton translocation. The proton pumping abilities of some bR analogs are compared. A visual pigment analog containing a nine-membered retinal has been prepared and studies such as FTIR are under study.

INTRODUCTION

Bacteriorhodopsin (bR), the protein found in the purple membrane of the archaebacterium Halobacterium halobium (refs. 1,2) functions as a light-driven pump and converts solar energy into chemical energy (ref. 3). It is the first membrane protein for which structural information could be obtained by electron microscope diffraction techniques (ref. 4), i.e., it has been shown to consist of 7 helical rods spanning the lipid bilayer. It is a protein comprised of 248 amino acids of known sequence (refs. 5,6) but the tertiary structure still remains to be settled (refs. 7,8).

The chromophore of bR is all-trans retinal covalently bound to lysine 216 via a protonated Schiff base (SB) linkage as determined by resonance Raman (refs. 9-11) and difference FTIR (refs. 12,13). Upon absorption of light, the light-adapted form of bR (bR_{LA}) is converted to the red-shifted photoproduct K, which according to vibrational spectral data, has a distorted 13-cis-retinal protonated Schiff base chromophore. The charge separation beween the positively charged nitrogen of the Schiff base and its negative counterion leads to energy storage (refs. 14-16), which induces a series of thermal relaxation of the pigment through intermediates L, M and O. The net result of the photo-induced cycle is the vectorial transport of protons from the cytoplasm to the external medium. A key intermediate in this proton translocation is M, the chromophore of which is now the deprotonated Schiff base of 13-cis retinal (ref. 17).

REVISED bR EXTERNAL POINT CHARGE MODEL

The protonated Schiff base (SBH+ or PSB) of retinal with n-butylamine absorbs at 445 nm (22,470cm^{-1} in MeOH) whereas bR, the chromophore of which is also a PSB with the terminal amino group of lysine 216, absorbs at 567 nm (17,640cm^{-1}). Similar red shifts are observed in the visual pigments, rhodopsins, which absorb in the range of 440-650 nm. The bathochromic shift, expressed in cm^{-1}, has been defined as the opsin shift (OS, ref. 18) and represents the overall environmental effect of the protein binding site on the absorption maxima of the pigments. The OS in the example shown is 4830cm^{-1} (Fig. 1). Theoretical treatment of the experimental maxima of a

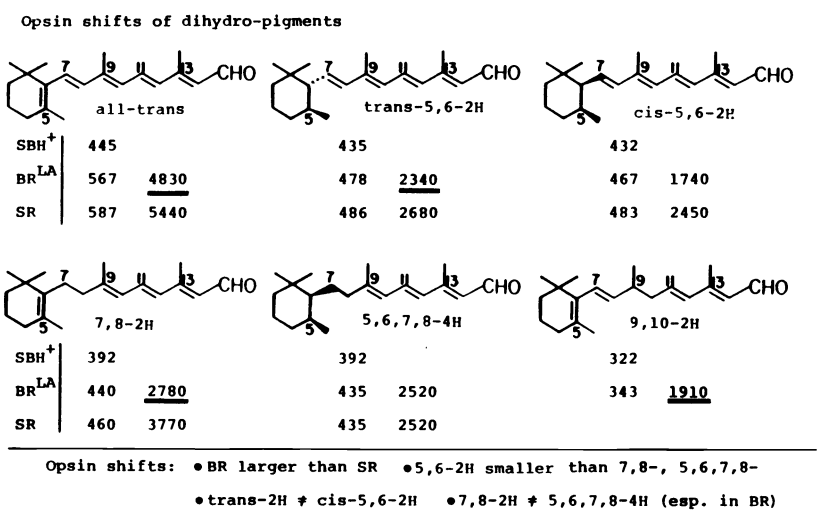

Fig. 1. Shifts of dihydrobacteriorhodopsin and dihydro-sensory rhodopsins.

series of pigment analogs derived from dihydroretinals led us to conclude that a major factor operating within the binding site was electrostatic interaction between the positively charged retinal moiety and two negatively charged groups of the protein, one near the nitrogen and the other near C-5 of the ionone ring (see Fig. 2, without the positive charge near C-1) (ref. 18). A model accounting for red shifts in the visual pigments was also forwarded (ref. 19).

However, the dihydro-bR series were re-examined in conjunction with recent incorporation experiments of dihydroretinals carried out with the sensory rhodopsin SR (ref. 20), upon which it was found that the previous OS for 7,8-dihydro-bR (7,8-2H-bR) required revision. The new set of OS values for bR^{LA} and SR are shown in Fig. 1.

In both pigments, the OS for trans-5,6-2H-bR 2340cm^{-1} is less than that of 7,8-2H-bR, 2780cm^{-1}; the same trend is seen for the cis-5,6-2H isomer and the SR analogs. This has led to the modified model shown in Fig. 2 where both members of the ion-pair interact with the ionone ring (ref. 21). The modified model incorporates a planar s-trans ring-chain conformation as has recently been suggested from NMR evidence (ref. 22). The similarity in the

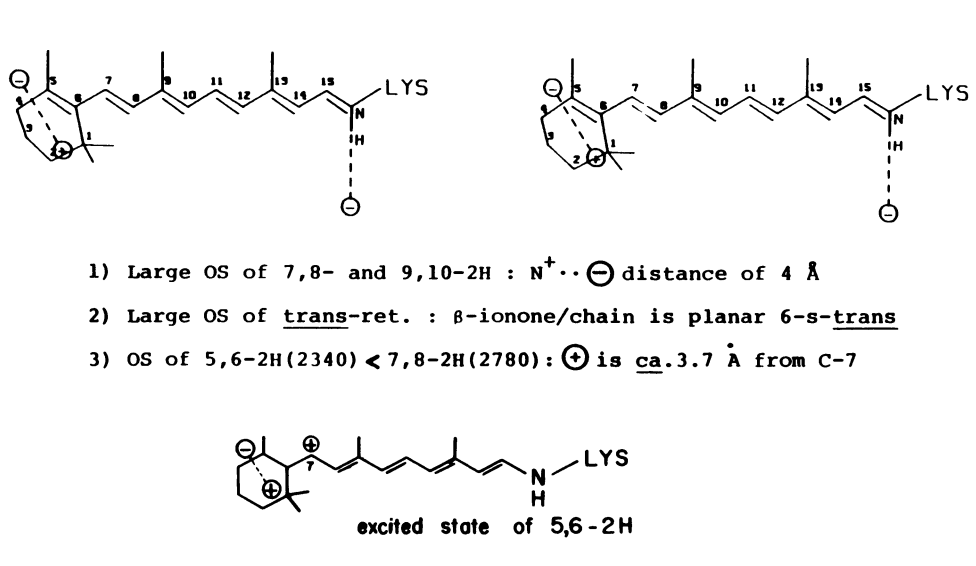

Fig. 2. Stereodrawing of bR and SR point charge models.

sets of data for bR and SR clearly show that the overall structures of the retinal binding sites in the two proteins are basically similar. The smaller OS of the 5,6-2H analogs as compared to the 7,8-2H analogs is now due to the positive end of the ion-pair being near the 7,8-double bond; this positive charge raises the energy of the excited state of the positively charged 5,6-2H- pigments. The data in Fig. 1 shows that the OS values of the trans- and cis-5,6-2H analogs, and of the 7,8-2H and 5,6,7,8-4H analogs differ, indicating that in agreement with recent studies (ref. 23), the stereochemistry of the chromophore plays an important role.

FTIR DIFFERENCE STUDIES OF bR

Resonance Raman spectroscopy (refs. 24-26) of biopolymeric pigments yields the vibrational spectra of the chromophore by selectively enhancing the Raman scattering of the prosthetic group. In contrast, IR spectroscopy measures vibrational spectra of the entire pigment including the protein and hence it is impossible to carry out subtle analyses, of the chromophore, e.g., the retinal moiety MW 285 in retinal pigments of 26,000 (bR) to 40,000 daltons (rhodopsins). However, by the difference technique, it becomes possible to detect vibrational modes of single amino acid residues as well as the chromophore, although the spectra have to be analysed with patience and caution because of the great complexity of bands (refs. 12, 13, 27-30).

In the case of the difference spectrum between bR and K intermediate, the steps involved are: (i) bR^{LA} spectrum is taken; (ii) photostationary state bR^{LA} + K is formed at 70K and the IR is taken; (iii) the difference of the two spectra will then give those modes that change between bR and K states. Measurements are done with hydrated films because bR does not function when dry; wet films are safe since experiments have shown that the kinetic behavior of wet films is similar to that of bR in action.

A typical difference spectrum is shown in Fig. 3; if the absorbance of the intermediate (K in this case) at a particular wavelength is greater than bR, the difference spectrum has a positive peak at that frequency. Conversely, if the bR absorbency is stronger, the spectrum has a negative peak.

In FTIR difference spectra of complex molecules, a powerful technique is to use isotopic shifts by modifying the chromophore and/or the protein. Usage of double isotope shifts offers a further advantage since assignment of a peak which appears in the isotope-containing pigment can be corroborated if that peak disappears (i.e., shifts further) upon further introduction of isotopes). This is exemplified below where the difference spectra of bR/K prepared from media containing tyr-d_0, tyr-d_2 (at o-positions to OH) and tyr-d_4 (at o- and m- positions to OH) are compared.

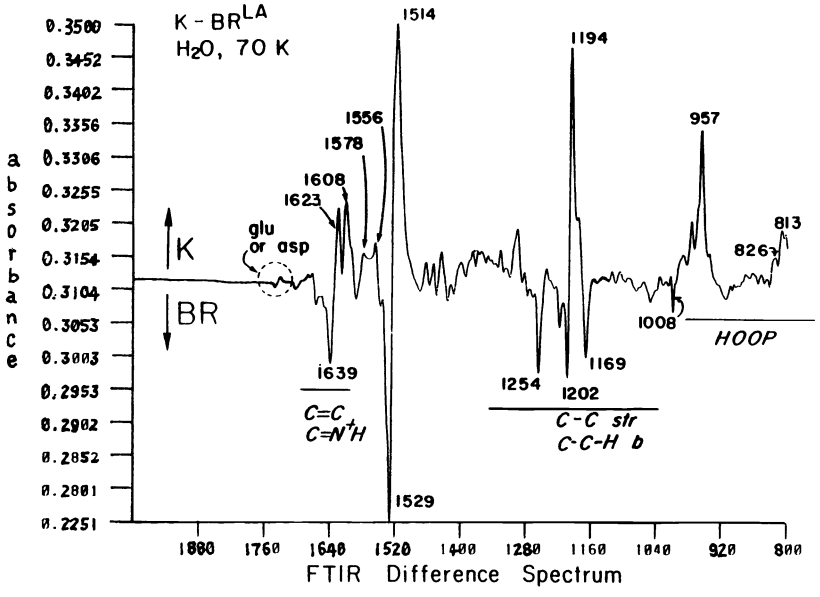

Fig. 3. FTIR difference spectrum between K (positive peaks) and bR (negative peaks).

In Fig. 3, the region around 1760cm^{-1} encircled "glu or asp" was clarified in the following manner. The weak negative (for bR) and positive peaks (for K) are at 1742 and 1732, respectively; the region is where monomeric carboxylic acid groups absorb. It indicates that either an asp or glu terminal COOH group is undergoing an environmental change in the bR to K photoisomerization. Since the difference peak positions remain unchanged in the pigments derived from 4-^{13}C-asp, the moiety affected is not asp but glu. Similar results employing 4-^{13}C-asp-bR have been published recently by Englehard, et al. (Ref. 31).

Similarly, the following changes were noticed: (1) the bR to L difference spectrum taken at 160K shows an intense 1740 bond for bR, suggesting that the asp residue(s) assigned to this band probably exists as a carboxylate in L. (2) Several asp and glu COOH groups are subject to environmental change in the bR to M conversion.

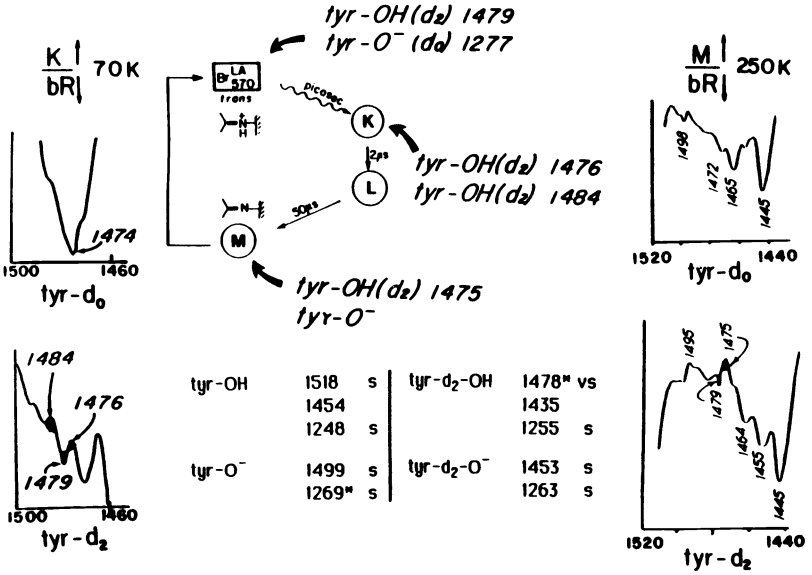

Fig. 4. FTIR difference spectrum of K-bR derived from tyr, tyr-d2 and tyr-d4.

Fig. 4 shows partial difference FTIR of the species K/bR and M/bR cultured in media containing regular tyr (tyr-d0), tyr-d2 (*ortho to OH group) and tyr-d4 (ortho and met to OH, spectrum not shown). The table shown in the fig-Fig. lists strong characteristics frequencies for tyrosine and tyrosinate. Although the tyr-d0 difference spectrum shows no band corresponding to these characteristic frequencies, the d2 spectra do show three lines at 1484-1476 suggesting they belong to the very strong 1478 band of tyr-d2; that this is so is verified by their disappearance in the tyr-d4 spectrum. Although not shown, the tyr-d0 K/bR has a 1277 line assignable to tyrosinate; this is absent in the d2 and d4 spectra. Likewise, the tyr-d2 M/bR measured at 250K shows lines at 1475 (form M) and 1479 (for bR), which again are absent in the d0 or d4 spectra. As summarized in Fig. 4, the tyr residues which undergo changes are: (i) in the bR to K transition a tyrosinate becomes protonated and the environment of one to three tyrosine changes; (ii) one of the tyrosines is deprotonated in M.

Further incorporation studies with 5-^{13}C-glu and others are being carried out with the purpose of clarifying the mechanism of the proton translocating phenomenon. Combination of FTIR and resonance Raman (RR) techniques offers powerful tools to study these dynamic processes.

PROTON PUMPING OF SOME bR ANALOGS

The bR analogs shown in Fig. 5 were incorporated into vesicles made from soybean phospholipids and the ability to pump protons across the lipid membranes were compared with reconstituted native bR. The trans and 13-cis-locked retinals (ref. 34), the "cyanine-dye" analog (ref. 35) and 7,8-2H, 9,10-2H analogs (ref. 30) and the 5-trifluoromethyl analog (ref. 37)

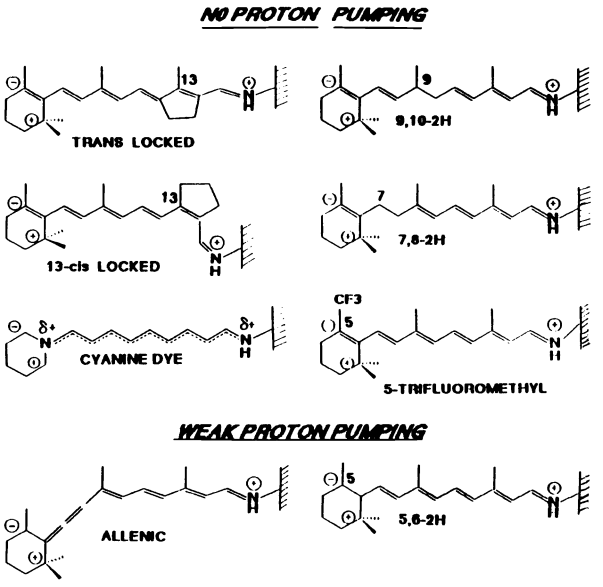

Fig. 5. Non-pumping and weakly pumping bR analogs.

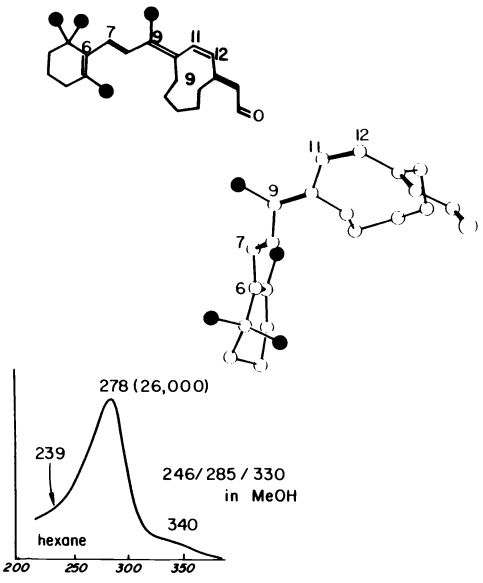

Fig. 6. Retinal with 9-membered ring in side chain and its UV.

do not translocate protons whereas the allenic and 5,6-2H analogs pump protons with 10-15% efficiency of native bR (Ref. 37). The results suggest that: (i) the full conjugated system is required for efficient functioning; (ii) interaction of the polyene with the ion-pair near the ionone ring may be necessary.

The 5-CF_3 analog presents intriguing results (ref. 37). Its maximum is at 465 nm leading to an OS value of only 2420 cm^{-1}; compare this with the OS of 4830 for native bR (Fig. 1). This can be accounted for by the model in Fig. 2 which places a negative charge near C-5; the strongly electronegative trifluoromethyl group is incompatible with this charge and hence the blue shift of the pigment. It was also found by resonance Raman studies (ref. 37) as well as HPLC extraction (see ref. 17a) that the light-adapted bR analog consists of 34:66 ratio of trans: 13-cis chromophores, whereas the dark-adapted (DA) species is 73:27 trans: 13 cis. In native bR the LA and DA species consist solely of trans and 13-cis chromophores. Also the LA species when left in the dark spontaneously changes into the DA pigment. Further studies of these phenomena should provide important clues regarding the mechanism of proton translocation.

RHODOPSIN WITH NINE-MEMBERED RETINAL ANALOG

The visual pigment rhodopsin also gives a primary photoproduct bathorhodopsin which corresponds to the K intermediate in bR. In an attempt to gain structural information on the K intermediate and also visual transduction, we have prepared an analog containing a nine-membered ring in the side chain (refs. 37, 38). The aldehyde depicted in Fig. 6 does form a visual pigment analog but the phenomenon is more complex than originally suspected. The process also is currently under study by difference FTIR.

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