Fourier Transform Infrared Study of the Halorhodopsin Chloride Pump[†]

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ABSTRACT: Halorhodopsin (hR) is a light-driven chloride pump located in the cell membrane of *Halobacterium halobium*. Fourier transform infrared difference spectroscopy has been used to study structural alterations occurring during the hR photocycle. The frequencies of peaks attributed to the retinylidene chromophore are similar to those observed in the spectra of the related protein bacteriorhodopsin (bR), indicating that in hR as in bR an all-trans → 13-cis isomerization occurs during formation of the early bathoproduct. Spectral features due to protein structural alterations are also similar for the bR and hR photocycles. For example, formation of the red-shifted primary photoproducts of both hR and bR results in similar carboxyl peaks in the 1730–1745-cm^{-1} region. However, in contrast to bR, no further changes are observed in the carboxyl region during subsequent steps in the hR photocycle, indicating that additional carboxyl groups are not directly involved in chloride translocation. Overall, the close similarity of vibrations in hR and bR photoproduct difference spectra supports the existence of some common elements in the molecular mechanisms of energy transduction and active transport by these two proteins.

Halorhodopsin (hR)[1] is a light-driven chloride pump found in the plasma membrane of *Halobacterium halobium* (Lanyi, 1986). In many respects, hR resembles the light-driven proton pump bacteriorhodopsin (bR) found in the purple membrane of the same organism. The two proteins have similar absorption spectra (Spudich & Bogomolni, 1983) and resonance Raman spectra (Smith et al., 1984; Maeda et al., 1985; Alshuth et al., 1985), the latter indicating that hR, like bR, has an *all-trans*-retinylidene chromophore with a protonated Schiff base. These proteins also exhibit similarities in the visible absorption spectra of the photocycle intermediates, although during its active chloride pumping cycle hR_{578} does not display an intermediate with a deprotonated Schiff base analogous to bR's M_{412} intermediate (Figure 1) (Lanyi, 1986). The amino acid sequence of hR (Blanck & Oesterhelt, 1987) also displays a high degree of homology with that of bR (Khorana et al., 1979), particularly in the internal lipophilic domains. Residues that are conserved include several near the retinal-binding Lys, four Trp, three interior Tyr, two interior Asp, and three Pro residues (Blanck & Oesterhelt, 1987). In view of these similarities, it is of interest to determine if bR and hR undergo similar structural alterations during their photochemical reaction cycles.

Fourier transform infrared (FTIR) difference spectroscopy is an effective method of probing small structural alterations in a protein, even at the single group level [for a recent review, see Braiman and Rothschild (1988)]. It has been used previously to study structural alterations in the bR photocycle (Rothschild et al., 1981; Rothschild & Marrero, 1982; Bagley et al., 1982; Siebert & Mäntele, 1983), providing evidence for protonation changes involving tyrosine and carboxyl groups (Engelhard et al., 1985; Rothschild et al., 1986; Dollinger et al., 1986; Roepe et al., 1987a,b) as well as changes in the environment of the Schiff base consistent with the proposed function of this group as a proton switch (Rothschild et al., 1984).

In the present study, hR was photolyzed at low temperature, and the resulting structural changes were studied by FTIR difference spectroscopy. The hR_{578} form of halorhodopsin, which is active in chloride pumping (Lanyi, 1986), was found to be present under the conditions of these experiments prior to illumination. It could be converted to a red-shifted form (hK_{630}) at 77 K and a blue-shifted form (hR_{520}) at 250 K. The spectral changes observed upon formation of hK_{630} were in some respects similar to those observed previously for K_{630}, the red-shifted photoproduct of bR, indicating that common structural alterations of the protein and chromophore occur during this early step in the two photocycles at low temperature. Similarities of protein structural changes are also observed at a later stage in the bR and hR photocycles. However in contrast to the bR photocycle, carboxyl changes do not appear to occur after the hR → hK step in the hR photocycle, possibly reflecting the different functions of these two proteins.

MATERIALS AND METHODS

Strain and Pigment Isolation. HR was purified from carotenoid-deficient bR$^-$ strain OD2W, derived by isolation of a spontaneous white colony from OD2 (Spudich & Spudich, 1982). Since the bR coding region of OD2W is inactivated by insertion of a transposable element within this region, the bR deficiency in this strain is absolute (DasSarma et al., 1983). Purification of hR was by modification of enrichment steps developed by Steiner and Oesterhelt (1983) and Taylor et al. (1983) as described (Hasselbacher et al., 1988). The hR preparation used here is capable of light-driven electrogenic transport when incorporated into asolectin liposomes (Bogo-

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[1] Abbreviations: FTIR, Fourier transform infrared; bR, bacteriorhodopsin; hR, halorhodopsin; PM, purple membrane.

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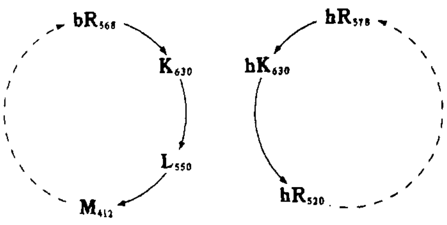

FIGURE 1: Photochemical reaction cycles of bR (left) and hR (right) showing the respective photointermediates and wavelengths of maximum visible absorbance.

molni et al., 1984; Hasselbacher et al., 1988).

FTIR Measurements. HR films were prepared by drying approximately 25 μL of the suspension of hR liposomes in 100 mM NaCl onto AgCl windows. Such films were found to exhibit a visible absorbance maximum near 578 nm, indicative of the chloride-bound form of hR (hR_{578}). HR films were then humidified by cooling the backside of the AgCl window with a stream of cold N_2 gas until water condensed on the film. The films were then sealed in a specially constructed cell which mounts in a HeliTran cryostat (Air Products, Allentown, PA).

HR films were light-adapted for 15 min with 500-nm light prior to cooling to either 77 K or 250 K. FTIR difference spectra were recorded on a Nicolet 60SX spectrometer (Madison, WI) with a protocol similar to that previously reported for bR (Rothschild et al., 1986; Roepe et al., 1987a,b). At 77 K, hR was illuminated alternately with 500- and 650-nm light. Completeness of photoreversal was determined by comparison of the intensity of peaks in FTIR difference spectra of successive forward and backward reactions. The first photoreversal was only 30% complete, but subsequent photoreversals were found to be at least 90% complete after 40 min of illumination at 650 nm.

At 250 K, the hR_{520} form was produced from hR_{578} by illumination with 600-nm light for 15 min. Reversal back to hR_{578} was found to be only partially complete after 15 min in the dark but could be completed when the dark period was followed by an additional 15 min of illumination with 450-nm light.

RESULTS AND DISCUSSION

$hR_{578} \rightarrow hK_{630}$. Figure 2 compares the FTIR difference spectra for the phototransitions of hR (A) and bR (B) at 77 K. As discussed below, the similarity of the spectra at 77 K argues strongly that similar structural alterations are occurring during the transition to the first metastable intermediate of their respective photocycles. The dominant feature in the hR → hK spectra is a negative peak at 1525 cm^{-1}. This frequency correlates well with the expected C=C stretching frequency for the retinylidene chromophore of hR_{578} on the basis of its absorption maximum (Aton et al., 1980) and is in agreement with reported resonance Raman spectra of this form of hR (Smith et al., 1984; Maeda et al., 1985; Alshuth et al., 1985). Under the conditions of our measurements, the formation of a red-shifted photoproduct with a 630-nm absorption maximum (Weber & Bogomolni, 1981) should result in a positive C=C stretching mode near 1514 cm^{-1}. A positive feature is observed in the $hR_{578} \rightarrow hK_{630}$ difference spectrum at this frequency, although it is much weaker than the corresponding peak in the $bR_{568} \rightarrow K_{630}$ spectrum. This may be due, however, to increased overlap with the negative peak at 1525 cm^{-1}.

A much stronger positive peak is observed at 1538 cm^{-1} which could also arise from an ethylenic mode of a second intermediate produced under these conditions. However, we consider this possibility unlikely since low-temperature visible absorption measurements made under identical conditions did

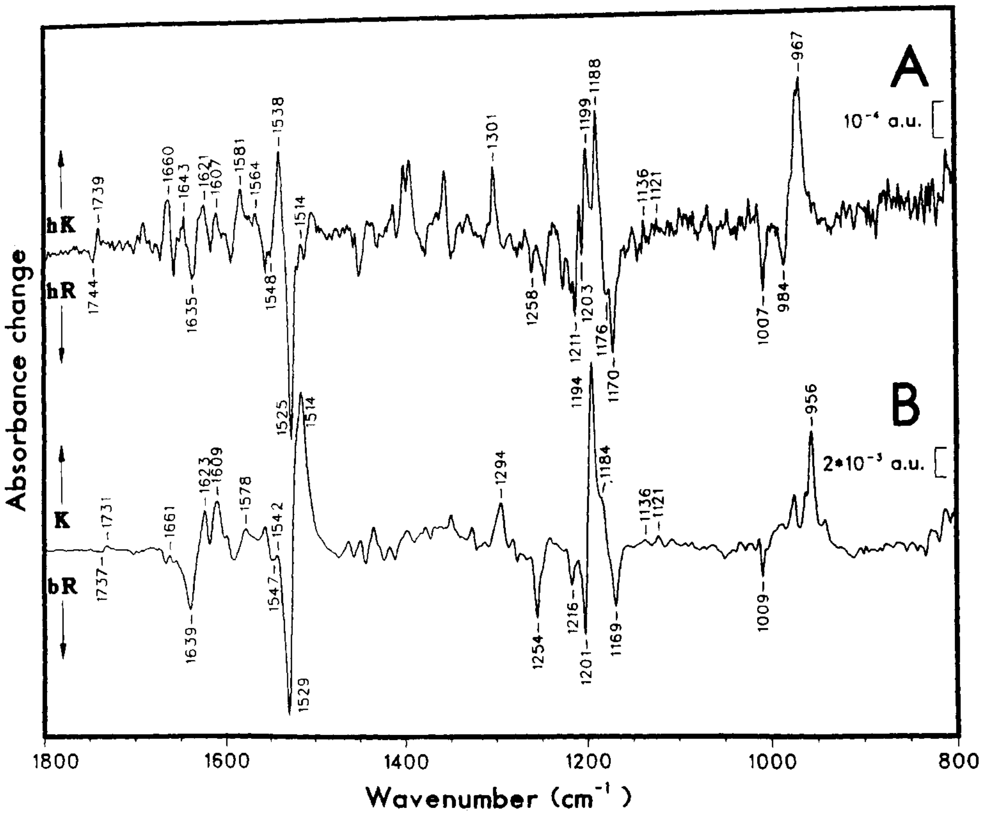

FIGURE 2: A comparison of (A) the $hR_{578} \rightarrow hK_{630}$ and (B) $bR_{570} \rightarrow K_{630}$ FTIR difference spectra at 81 K recorded at 2-cm^{-1} resolution. Green illumination (Ditric interference filter 15-11180, Hudson, MA) was used to drive hR_{578} into the hK_{630} state, and red illumination (Ditric interference filter 15-11200) was used to photoreverse hK_{630} to hR_{578}. The conditions of the bR measurements were similar to those reported previously (Rothschild et al., 1986). Longer illumination times of 40 min were utilized in the case of hR as compared to 20 min for bR in order to obtain complete photoreversal.

not reveal such a species, which would be expected to absorb near 550 nm (O. Bousché, K. J. Rothschild, and J. L. Spudich, unpublished data). Alternatively, the 1538-cm^{-1} peak could arise from alterations in the amide II band of the peptide backbone. A similar protein change has been recently identified in the rhodopsin → bathorhodopsin transition in visual pigment (Rothschild & DeGrip, 1986).

The C=N stretching vibrations of hR and bR can be assigned at 1635 and 1639 cm^{-1}, respectively, on the basis of resonance Raman measurements (Smith et al., 1984; Maeda et al., 1985; Alshuth et al., 1985). The lower frequency in hR has previously been attributed (Smith et al., 1984) to a weaker coupling with the NH in-plane rock in hR relative to bR, possibly as a result of a weakening in the hydrogen bonding between the Schiff base proton and a counterion. The assignment of the Schiff base frequency of hK_{630} is less certain, although close agreement is found in the 1609-cm^{-1} region where the C=N stretching mode for K_{630} has previously been assigned (Rothschild et al., 1984). In fact, the 1607-cm^{-1} peak due to hK_{630} disappears in D_2O-humidified films (data not shown), supporting the assignment of this peak to a protonated Schiff base. However, a more extensive set of isotopic substitutions will be necessary to make the assignment definitive and to determine where this peak may shift. The similarity of the absorption maxima of these two early photointermediates from hR and bR also supports the existence of a similar environment for the C=N group in both K_{630} and hK_{630}.

It is notable that if one takes into account the above-mentioned differences in frequency of the C=N and C=C stretches, almost all of the remaining peaks above 1500 cm^{-1} are at a very similar frequency in the hR and bR difference spectra at 77 K, although the intensities are quite different. In the $bR_{568} \rightarrow K_{630}$ spectrum, it is known that these peaks are mainly due to protein vibrations. For instance, many of the peaks in the 1600–1700-cm^{-1} region of the bR → K and the hR → hK difference spectra are presumably due to amide I modes (carbonyl stretch of peptide groups) (Fraser & McRae, 1973) and reflect small structural changes in the protein backbone. Even the positive peak found at 1643 cm^{-1} in the hR → hK difference spectrum appears in the bR difference spectrum when the negative C=N band at 1640 cm^{-1} is shifted away by isotope labeling (Earnest and Rothschild, unpublished data). A pair of peaks in the range 1535–1550 cm^{-1}, which may be due to changes in the peptide amide II modes, is also present in both the hR → hK and bR → K difference spectra, although they are weaker in the latter. Overall, the similarity of peak frequencies in the amide I and II regions indicates that the two proteins undergo similar protein conformational alterations early in their respective photocycles, although differences in intensities must be taken into account. One possible cause of intensity differences is a different degree of orientation of the bR and hR protein relative to the IR beam polarization. In particular, the amide I region of the bR → K difference spectrum exhibits intensified peaks, especially the 1661-cm^{-1} band, when recorded with infrared light polarized out of the sample plane (Earnest et al., 1986).

A pair of peaks in the 1730–1745-cm^{-1} region appears in both the $bR_{568} \rightarrow K_{630}$ and $hR_{578} \rightarrow hK_{630}$ difference spectra. In the bR case, these peaks (negative peak at 1737 cm^{-1}; positive peak at 1731 cm^{-1}) have been previously assigned to changes in either the hydrogen bonding or the protonation of one or more Asp carboxylic acid groups (Engelhard et al., 1985). Similar peaks have also been observed in the difference spectra of the primary phototransition in the photocycle of dark-adapted bR ($bR_{548} \rightarrow K^{D}_{610}$) (Roepe et al., 1988), which involves chromophores with different $C_{13}=C_{14}$ and $C_{15}=N$ bond configurations than in bR_{568} and K_{630}. In the case of hR, the two carboxyl groups which could give rise to this dispersion are Asp-248 and Asp-141, which are conserved in bR as Asp-212 and Asp-115. Since recent FTIR studies on mutants of bacteriorhodopsin (M. Braiman, H. Khorana, B. Chao, L. Stern, T. Mogi, and K. Rothschild, unpublished data) indicate that in the bR case the dispersion is not due to Asp-212, the signals may arise from Asp-115, which has been predicted to serve as the counterion near the β-ionone ring of retinal (Renthal et al., 1985), and thus could be in a position to be perturbed by formation of a red-shifted intermediate possibly due to charge redistribution in the chromophore. The presence of a negative charge near the C-5 of the ring has been inferred from absorption spectroscopy of bR containing analogues (Nakanishi et al., 1980) and from NMR results for both bR_{568} and bR_{548} (Harbison et al., 1985). Thus, a perturbation of Asp-115 (bR) or the homologous residue Asp-141 (hR) may explain the carboxyl signals detected during formation of the photoproducts hK_{630}, K_{630}, and K^{D}_{610}.

It can also be concluded from the 77 K difference spectra that the retinylidene chromophores of bR and hR undergo similar structural changes in the early photocycle. It has already been established on the basis of resonance Raman measurements that the chromophores in bR_{568} and hR_{578} have an all-trans configuration (Braiman & Mathies, 1980; Smith et al., 1984) and that isomerization occurs about the $C_{13}=C_{14}$ bond during the $bR_{568} \rightarrow K_{630}$ photoreaction (Braiman & Mathies, 1982; Braiman, 1983). In the hR difference spectrum, the negative peaks at 1176, 1203, 1211, and 1258 cm^{-1} are in good agreement with the frequencies of the predominantly C–C stretching modes appearing in the resonance Raman spectrum of hR_{578} (Smith et al., 1984). Positive peaks of hK_{630} at 1121, 1136, 1188, and 1199 cm^{-1} (Figure 2A) agree well with the positive peaks of K_{630}, the last two assignable to C–C stretching modes (Braiman, 1983; Smith et al., 1986). We also note that hK_{630} displays an intense peak at 967 cm^{-1} which could originate from a HOOP mode observed in K_{630} at 956 cm^{-1}. Thus, the vibrational spectrum of the hK_{630} chromophore resembles that of K_{630}, and it is therefore likely to be in a distorted 13-cis configuration as well. Finally we note that there are several prominent bands in the hR → hK difference spectrum, especially in the 1300–1400-cm^{-1} region, which are not yet assigned to protein or chromophore vibrations and do not agree with the bR → K difference spectrum. Isotopic labels introduced into the chromophore and protein of hR will facilitate assignment of these peaks.

$hR_{578} \rightarrow hR_{520}$. Evidence for a 13-cis form of the late hR photocycle products has been obtained in several laboratories (Ogurusu et al., 1981; Lanyi, 1984; Oesterhelt et al., 1986). Both Diller et al. (1987) and Fodor et al. (1987) have found from resonance Raman studies that the chromophore configuration and environment of the hR_{520} photointermediate and the L_{550} photointermediate of bR are almost identical. By comparing the difference spectra for hR ($hR_{578} \rightarrow hR_{520}$) and bR ($bR_{568} \rightarrow M_{412}$) recorded at 250 K (Figure 3A,B), we now find that the protein structural rearrangements, especially in the peptide backbone, are similar in the two proteins. In contrast, the $bR_{568} \rightarrow L_{550}$ difference spectrum (Figure 3C) 170 K did not exhibit as great a similarity to the $hR_{578} \rightarrow hR_{520}$ spectrum (cf. Figure 3A).

In the amide I region between 1650 and 1700 cm^{-1}, peaks appear at similar frequencies in both the $hR_{578} \rightarrow hR_{520}$ and $bR_{568} \rightarrow M_{412}$ difference spectra. However, their relative

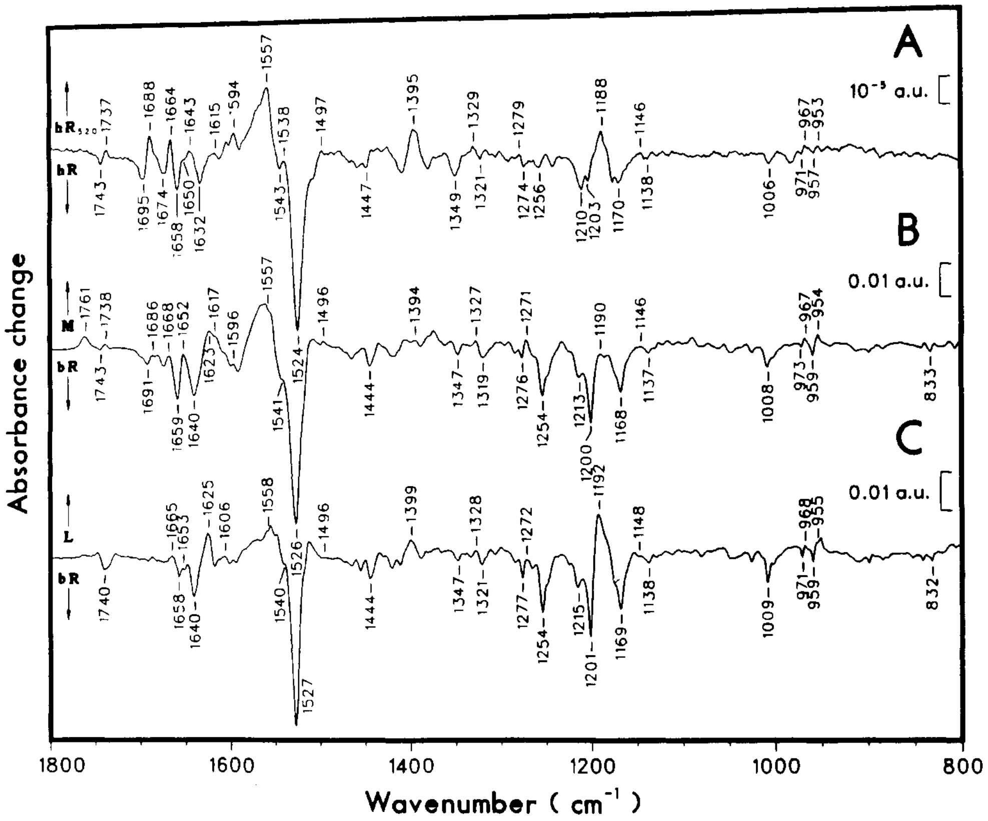

FIGURE 3: A comparison of the FTIR difference spectra for (A) $hR_{578} \rightarrow hR_{520}$ at 250 K, (B) $bR_{568} \rightarrow M_{412}$ at 250 K, and (C) $bR_{568} \rightarrow L_{550}$ at 170 K. Spectra B and C were recorded under conditions reported in Roepe et al. (1987a). See text for details of spectrum A.

intensities differ somewhat, an effect which might be due to the intrinsic IR dichroism of many of these peaks as observed in bR (Earnest et al., 1986). Also, the differences between the two spectra in the 1640–1630-cm^{-1} range can be accounted for by different C=N stretch frequencies in hR and bR and their corresponding intermediates (Diller et al., 1987). Note that many of the common peaks in the 1650–1700-cm^{-1} region for the transitions of hR and bR at 250 K do not appear or are greatly reduced in intensity in the case of the $bR_{570} \rightarrow L_{550}$ differences (Figure 3C).

In the amide II region (1530–1560 cm^{-1}), the peak at 1557 cm^{-1}, which increases in intensity for bR at higher temperatures (Braiman et al., 1987; Marrero & Rothschild, 1987), most likely reflects a change in the peptide backbone. In the case of $hR_{578} \rightarrow hR_{520}$ this region is also expected to contain a contribution from the C=C stretch of the hR_{520} intermediate near 1552 cm^{-1} (Diller et al., 1987) which is not resolved. The small inflection near 1540 cm^{-1} is also present in both proteins; it may arise from changes in the amide II mode which persist from the K_{630} and hK_{630} intermediate states (see above).

The lack of new features in the carboxyl region above 1700 cm^{-1} in the $hR_{578} \rightarrow hR_{520}$ spectrum compared to the $hR_{578} \rightarrow hK_{630}$ spectrum (cf. Figure 1) indicates that hR differs from bR in that, after the initial step of the photocycle, no additional changes occur in Asp or Glu groups.[2] This finding may be directly related to the different functions of hR and bR. In the case of bR, the carboxyl changes in this region which occur after K_{630} formation have been postulated to be involved in proton transport and in addition may reflect the protonation of a carboxylate group which serves as the acceptor for the Schiff base proton (Rothschild et al., 1981; Engelhard et al., 1985; Dollinger et al., 1986; Roepe et al., 1987a). In contrast, the movement of chloride ions may not involve an obligatory proton movement, and in addition, the absence of a Schiff base deprotonation in hR removes the requirement of a negative acceptor group. Despite these difference, the similarities between the chromophore and protein skeletal changes which occur in bR and hR during the photocycle still suggest the overall mechanism operative in the two different transport proteins is very similar.

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[2] Since these measurements were made at 250 K, it is possible that additional carboxyl changes can occur at physiological temperatures. Time-resolved FTIR can be utilized to check this possibility (Braiman et al., 1987).

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