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EVIDENCE FOR LIGHT-INDUCED LYSINE CONFORMATIONAL CHANGES DURING THE PRIMARY EVENT OF THE BACTERIORHODOPSIN PHOTOCYCLE

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Fourier transform infrared difference spectroscopy is used to examine the role of lysine in the primary event of the bacteriorhodopsin photocycle. Isotopically labeled lysine is used to tentatively assign the lysine modes in the BR and K species. The results suggest that the lysine side-chain undergoes conformational changes in concert with the known light-induced chromophore structural alterations.

Bacteriorhodopsin (BR)[1] is a 26,000-dalton pigment in the purple membrane (pm) of *Halobacterium halobium*. BR is composed of an all-trans retinal chromophore bound via a protonated Schiff base to the ε-amino group of a lysine residue of the protein bacterio-opsin. This protein consists of alpha helices that traverse the membrane seven times with the retinal linked to the lysine residue numbered 216 (Lys-216) on the C-terminal helix. Absorption of a visible photon by the retinal chromophore of BR provides the energy which is transduced into the movement of protons across the bacterial cell membrane. The resulting electrochemical gradient is coupled to the synthesis of ATP. The absorption of a photon initiates a series of mutual structural changes in the retinal chromophore and the protein. These changes form a photocycle composed of a series of intermediates (for a review, see reference 1).

The BR photocycle has been established by time-resolved visible absorption spectroscopy. The primary photoproduct is K_{610}[2] followed by the intermediates L_{550}, M_{412}, N_{520} and O_{640}; returning to BR_{570} in ~10ms (2,3). Resonance Raman spectroscopy has been useful in elucidating the retinal chromophore structure and the Schiff base protonation state for each intermediate (4-8). Fourier transform infrared (FTIR) difference spectroscopy has supported many of the conclusions deduced from resonance Raman spectroscopy (9-11). Whereas resonance Raman spectroscopy selectively probes vibrational modes directly coupled to the electronic transition, FTIR difference spectroscopy detects all the IR active modes that change with light. Thus, in addition to detecting retinal alterations, FTIR difference spectroscopy has been able to probe protein structural changes induced by light. Studies on specific amino acids of BR have monitored the protonation state of aspartic acid, glutamic acid, and tyrosine residues (12-17). We report here the first results from FTIR difference spectroscopy which directly probe side-chain vibrational modes of lysine residues in the

[1] Abbreviations: BR = bacteriorhodopsin, pm = purple membrane, FTIR = Fourier transform infrared.

[2] The subscript indicates the visible absorption maximum in nanometers. The K intermediate has a visible absorption maximum near 610nm at room temperature and near 630nm at 80K.

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BR_{570} and K_{630} species. Our results indicate that, in concert with the well known chromophore structural alterations, there are light-induced conformational changes in lysine.

Materials and Methods

Materials D,L-lysine-3,3,4,4,5,5,6,6-[$^2H_8$] 2HCl (98.8 atom % ^2H) was purchased from MSD Isotopes (Montreal, Quebec) and used without further purification. Native pm was prepared by growing Halobacterium halobium S9 on a modified Onishi medium (18). [$^2H_8$]lysine pm was prepared using an identical procedure except that twice the quantity of D,L-lysine-3,3,4,4,5,5,6,6-[$^2H_8$] 2HCl was substituted for L-lysine HCl. Little, if any, deuterium from the deuterated lysine transfers to other amino acid residues and bacterial synthesis of unlabeled lysine is unlikely (19). Purple membrane (pm) sheets were isolated and washed several times in H_2O.

Wet films were made by depositing several drops of concentrated pm (A_{570}~4, 1 cm pathlength) on a AgCl window (International Crystal Laboratories, Inc., Elizabeth, NJ) and partially drying using N_2 gas. The sample was sealed by adding a second AgCl window with a thin Ag O'Ring and placed in a cryocooler (Cryogenic Technology, Inc., Waltham, MA) suspended in an FTIR spectrometer (IR/98, IBM Instruments Inc., Danbury, CT), equipped with a liquid N_2 cooled HgCdTe detector. Water content of the film was monitored by comparing the integrated intensity of the water band at 3300 cm^{-1} to the amide I band. Ratios of approximately 6 to 1 or higher were considered adequately hydrated (16).

FTIR Spectroscopy The sample is light adapted with yellow light (λ>515nm filter, Esco Products Inc., Oak Ridge, NJ) for at least 15 min. at room temperature and then cooled in the dark to 80K. An FTIR difference spectrum is obtained by:

1) Collecting an FTIR spectrum of BR in the dark (512 scans, 2 cm-1 resolution, ~7 min.)
2) Shining 500 nm light (40 nm bandpass filter, Corion Corp., Holliston, MA) for 10 minutes.
3) Collecting an FTIR spectrum of BR+K in the dark (512 scans, 2 cm-1 resolution, ~7 min.)
4) Subtracting the BR spectrum from the BR+K spectrum to produce a difference spectrum.
5) Shining ~650 nm light (650 nm long-wavelength pass filter in series with an IR absorbing filter (Melles Griot, Irvine, CA)) for ~15 minutes to regenerate BR.

This procedure is repeated at least four times and the resulting difference spectra are averaged to produce a K/BR difference spectrum for both native and [$^2H_8$]lysine pm. The individual difference spectra are highly reproducible in terms of both frequency and relative intensity. Every peak labeled in the results and discussion section is clearly seen in each of the individual difference spectra. The spectra shown are unsmoothed with a resolution of 2 cm^{-1}. The Fourier transforms were computed using Happ-Genzel apodization.

Results and Discussion

Determination of lysine peaks in the K/BR FTIR difference spectrum. In order to detect the vibrational modes in BR_{570} and K_{630} which are due to lysine, FTIR difference spectra were obtained at 80K for hydrated films of both native pm and [$^2H_8$]lysine pm. At this temperature the primary photoproduct, K_{630}, is indefinitely stable and light activated pm samples contain only the species BR_{570} and K_{630}. The K/BR difference spectra obtained for the native pm sample and the [$^2H_8$]lysine pm sample are shown in Figures 1A and 1B, respectively. In these spectra positive peaks originate from K_{630} and negative peaks are due to BR_{570}. The majority of K_{630} and BR_{570} peaks in the two spectra are very similar in intensity and frequency. For example, Figure 1 shows the C=N-H^+ stretching vibration of the Schiff base does not change by more than 2 cm^{-1} as a result of [$^2H_8$]lysine substitution. In fact, most of the clearly identifiable retinylidene chromophore vibrations (e.g. C=N, C=C and C-C stretches) are completely unchanged in frequency and relative intensity by substituting [$^2H_8$]lysine in place of the native amino acid. However, there are numerous bands that either

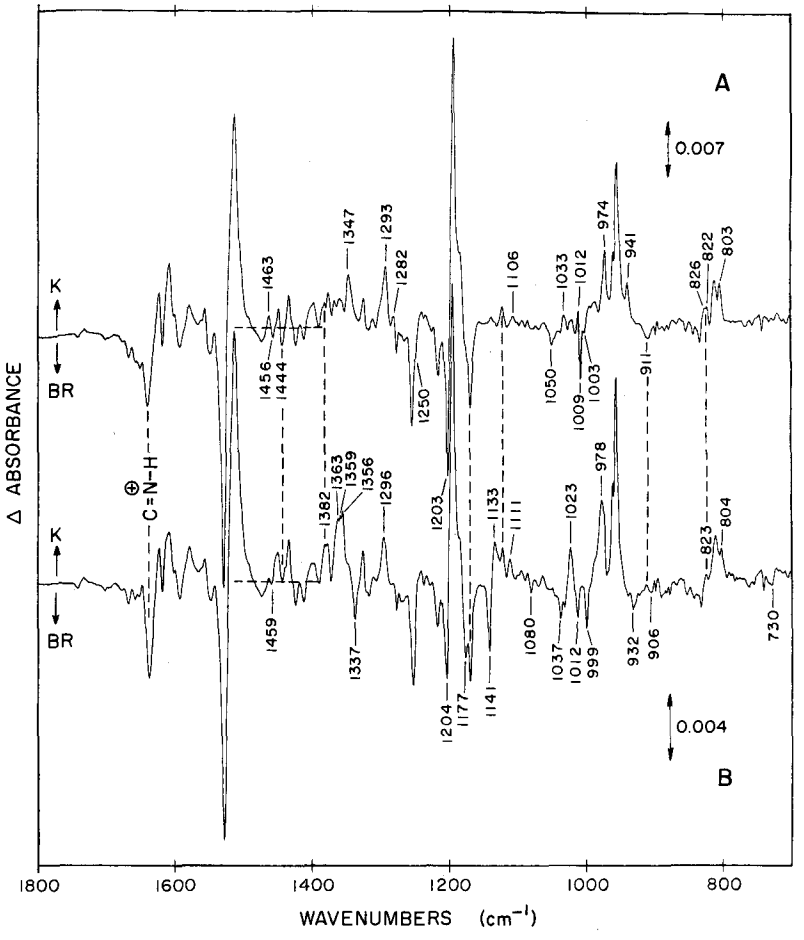

Figure 1. K/BR Fourier transform infrared difference spectra in the range 700 - 1800 cm^{-1} at 80K and 2 cm^{-1} resolution in H_2O. (A) Native purple membrane film. (B) [$^2H_8$]lysine purple membrane film. The arrow indicates the absorbance scale.

completely disappear or are significantly altered by the above replacement. These bands are labeled with their frequencies in Figure 1. All labeled peaks have either reproducibly different frequencies (>2 cm^{-1} difference) or clearly different intensities in the two spectra of Figure 1. The spectral differences can be caused by isotope-induced shifts of lysine vibrational modes in or out of a particular region and/or changes in non-lysine vibrational modes via an isotope-induced alteration in coupling to lysine.

Assignment of lysine modes in the K/BR FTIR difference spectrum. As a first approximation, a vibrational mode may be associated with local symmetry coordinates of the molecule. For the side-chain of lysine the local symmetry coordinates are the CH_2 scissor, CH_2 wag, CH_2 twist, CH_2 rock, C-N stretch and the C-C stretch. Table 1 lists the vibrational frequency range found for each of these modes in fully protonated and fully deuterated compounds similar to lysine.

The region 1438 - 1489 cm^{-1} has been assigned to CH_2 scissor modes (see Table 1). We notice significant alterations in this region as a result of the [$^2H_8$]lysine substitution. A horizontal dashed line has been added in this region as an aid in differentiating positive from negative peaks. Upon

Table 1. The range of vibrational frequencies observed for some modes appropriate to lysine. The data are from analyses on poly-L-lysine (20), n-butylamine (21), n-propylamine (22), hexane, pentane, and butane (23) for the fully protonated compounds and from hexane and pentane (23) for the fully deuterated compounds.

MODE	FREQUENCY RANGE (cm^{-1})	
	Fully Protonated	Fully Deuterated
CH_2 scissor	1438 - 1489	968 - 1152
CH_2 wag	1246 - 1410	727 - 893, 987 - 1042
CH_2 twist	1180 - 1342	726 - 794, 933 - 967
CH_2 rock	725 - 910, 1131 - 1179	520 - 659, 980 - 991
C-N stretch	1030 - 1141	—
C-C stretch	811 - 1124	709 - 892, 1115 - 1246

[$^2H_8$]lysine substitution the 1463 cm^{-1} K_{630} band and the 1444 cm^{-1} and 1456 cm^{-1} BR_{570} bands in native pm have lost intensity. If these are indeed lysine CH_2 scissor modes they would shift to the CD_2 scissor region (968 - 1152 cm^{-1}) upon deuteration (see Table 1). In this region of the [$^2H_8$]lysine pm spectrum we observe numerous new K_{630} and BR_{570} bands which are possible candidates for CD_2 scissor modes.

According to Table 1, CH_2 wag and CH_2 twist modes may be found in the 1180 - 1410 cm^{-1} region of the native pm spectrum. In this region is an intense positive peak at 1347 cm^{-1} which is not found in the deuterated spectrum. According to Table 1, this may be a CH_2 wag mode. If this is the case, the intense positive peak at 1023 cm^{-1} in the [$^2H_8$]lysine pm spectrum is a likely candidate for the shifted CD_2 wag mode. Alternatively, the 1347 cm^{-1} peak has been assigned as an N-H in-plane bending mode (11). The intense positive peak at 1293 cm^{-1} in the native spectrum is probably a chromophore peak which shifts to 1296 cm^{-1} upon [$^2H_8$]lysine substitution.

Other possible CH_2 wag and/or CH_2 twist modes of the lysine side-chain are indicated by losses of intensity at 1282, 1250, and 1203 cm^{-1} in the native pm spectrum. According to Table 1, the corresponding peaks in the [$^2H_8$]lysine pm spectrum may be those at 730 cm^{-1} or in the region from 999 to 1037 cm^{-1} (see Figure 1B).

The 1180 - 1410 cm^{-1} region of the [$^2H_8$]lysine pm spectrum is composed of a triplet of intense peaks between 1356 and 1363 cm^{-1}, flanked by a negative peak at 1337 cm^{-1} and a gain of intensity at 1382 cm^{-1}. The frequencies of these modes are too high to be CD_2 modes and their assignment is, at present, unclear.

We find several candidates for C-N and/or C-C stretch modes in the 1030 - 1141 cm^{-1} region of the native pm spectrum. These occur as K_{630} bands at 1106 and 1033 cm^{-1} and as a BR_{570} band at 1050 cm^{-1}. In the C-C stretch region, a whole sequence of changes due to [$^2H_8$]lysine substitution occur between 1012 and 822 cm^{-1}. Among these are alterations in K_{630} bands at 1012, 974, and 941 cm^{-1} and in BR_{570} bands at 1009, 1003 and 911 cm^{-1}. If these are C-C stretch modes they should either shift up to the 1115 - 1246 cm^{-1} region or down to the 709 - 892 cm^{-1} region upon [$^2H_8$]lysine substitution (see Table 1). Within these regions of the [$^2H_8$]lysine pm spectrum we find new intensity at 1177, 1141, 1133 and 730 cm^{-1}.

The K_{630} peaks at 826, 822, and 803 cm^{-1} are in the region where CH_2 rock modes can occur. If these are CH_2 rock modes, they would most likely shift to the 520 - 659 cm^{-1} CD_2 rock region upon [$^2H_8$]lysine substitution. Unfortunately, our HgCdTe detector cuts off at about 700 cm^{-1}.

Table 2. Tentative assignments for lysine modes in the K/BR difference spectrum of native purple membrane in H_2O (see Figure 1A)

BR_{570}	K_{630}	MODE
1456	1463	CH_2 scissor
1444		"
	1347	CH_2 wag
1250	1293	CH_2 wag, CH_2 twist
	1282	"
1203		CH_2 twist
1050	1106	C-N st., C-C st.
	1033	"
1009	1012	C-C stretch
1003	974	"
911	941	"
	826	C-C st., CH_2 rock
	822	"
	803	CH_2 rock

A complete summary of our tentative assignments for the lysine vibrational modes of the K_{630} and BR_{570} species in native purple membrane appears in Table 2. The reader is reminded that some of the peaks in the K/BR FTIR difference spectrum, which we assigned to lysine, may be chromophore modes coupled to lysine.

From Figure 1 it is apparent that certain regions of the spectrum, such as the 1500-1800 cm^{-1} regime, are completely unaltered by [$^2H_8$]lysine substitution. These regions are clearly assigned to the double bond stretches of the retinylidene chromophore and certain amino acid side-chains (e.g. aspartic acid, glutamic acid and arginine). On the other hand, frequency regimes discussed above where significant alterations are observed by deuterium substitution are associated with previously identified lysine side-chain modes. Although this is encouraging, deuteration of simple molecules indicates that no bands should be observed in certain regions where [$^2H_8$]lysine pm samples show intense vibrations. The precise reason for this observation in not clear. Nonetheless, the simplest explanation of our data is that upon light-induced transformation from BR_{570} to K_{630}, not only the retinylidene chromophore structure is altered but, in addition, the lysine structure is also affected. It is plausible to assume that, in view of the rapid timescale of K_{630} production, the lysine vibrations that are altered are associated with the lysine-216 side chain attached to the retinylidene chromophore. At the present time this is simply an hypothesis. Additional experiments will allow us to test this hypothesis by investigating FTIR difference spectra of bacteriorhodopsin permethylated (24) at all of the lysines other than lysine-216.

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