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CONFORMATIONAL CHANGES OF BACTERIORHODOPSIN DETECTED BY FOURIER TRANSFORM INFRARED DIFFERENCE SPECTROSCOPY

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SUMMARY: We report the first Fourier transform infrared difference spectra of purple membrane. Evidence is presented that alterations in the vibrations of both the retinylidene chromophore and the protein groups of bacteriorhodopsin associated with photocycling can be detected. This method provides a new tool for probing the conformational changes occurring in bacteriorhodopsin during the proton pump cycle.

INTRODUCTION: Bacteriorhodopsin, the light-activated proton pump from the purple membrane of *Halobacterium halobium*, is a highly attractive system for studying the mechanism of energy transduction and ion transport in biological membranes (1). The three-dimensional structure of bacteriorhodopsin is known on the basis of electron microscopic diffraction to 7 A and reveals seven alpha-helical segments which are oriented perpendicular to the membrane plane (2). The primary sequence of bacteriorhodopsin is established (3) and with this information the approximate position of the peptides through the membrane has been predicted (4). Chemical modification of specific amino-acids in the sequence has also begun to be used in conjunction with transport studies to determine the role of specific protein groups (5).

In order to further elucidate the mechanism of proton transport in bacteriorhodopsin it will be necessary to obtain information about the molecular changes occurring during the photocycle. In this regard resonance Raman spectroscopy provides a selective probe of the retinylidene chromophore vibrations (6-10). We have recently begun to apply infrared spectroscopy to probe bacteriorhodopsin structure (11,12). In contrast to resonance Raman spectroscopy, infrared absorption is sensitive to vibrations from all components of the purple membrane. Furthermore, it is possible to assign peaks in the infrared spectrum to specific protein and lipid

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groups and in some cases determine their net orientation relative to the membrane plane (11). It would be advantageous to measure also the infrared absorption changes occurring in the vibrational modes of purple membrane during the photocycle, since, in contrast to resonance Raman, infrared light does not drive the photocycle. It is difficult, however, to detect changes in the infrared spectrum related to bacteriorhodopsin photocycling using a conventional IR scanning spectrometer since these changes are extremely small . For this reason we have utilized Fourier transform infrared spectroscopy (13), which enables high signal/noise spectra to be recorded over a large region in less than one minute. The sensitivity of this technique makes it possible to study small conformational changes involving single groups in the protein.

We present in this paper Fourier transform infrared difference spectra which reflect the molecular changes occurring in bacteriorhodopsin upon illumination. By using isotope labeling it is possible to distinguish between vibrational changes associated with the protein and the chromophore. We find that changes in the C=C stretching mode of the retinylidene chromophore appears as one of the largest features of the spectrum.

MATERIALS AND METHODS: Purple membrane was isolated from Halobacterium halobium originally derived from strain S9 using the method of Becher and Cassim (14). Thin films formed from suspension of purple membrane in distilled H_2O were prepared on AgCl windows or plastic microscope coverslips (Fisher Scientific, N.J) using the isopotential spin-dry method reported on in detail elsewhere (15,16). The optical density of these films at 560 nm varied between .3-.5 O.D. For the purpose of visible absorption spectroscopy, films on plastic were mounted in disposable cuvettes containing a small amount of drierite at the bottom and sealed at the the top with parafilm. Films were equilibrated for a minimum of 2 hours prior to measurement. Such dried films had a maximum absorption at 560 nm and exhibited no light adapted state at 568 nm in agreement with the studies of Korenstein and Hess (17,18). Films were illuminated for 1 minute with a 600 watt tungsten source filtered by a #12 Wratten filter and an IR heat filter and then scanned in the dark at two minute intervals using a Cary 219 UV-Visible absorption spectrometer. The difference spectrum indicated in agreement with refs. 17 and 18 that the M412 intermediate is predominantly formed during prolonged illumination with no evidence of a buildup of the O660 intermediate from the light cycle or C610 species from the dark cycle. The time constant for decay of M412 in dehydrated films was around 4 minutes.

Infrared measurements of purple membrane film deposited on AgCl windows were made with a Nicolet MX-1 FTIR spectrometer. The sample compartment was purged with dry N_2 and the film allowed to equilibrate for a minimum of 1 hour. Spectra were recorded prior, during and after illumination of the sample using the same 600 watt filtered tungsten source described above for visible absorption measurements. A spectrum normally consisted of the average of 32 scans which took one minute to

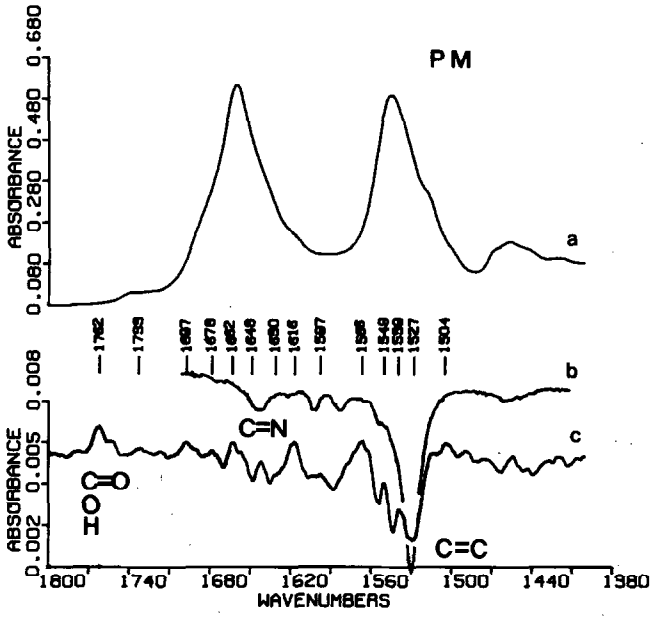

Figure 1. (a) Infrared absorption spectrum of a purple membrane film deposited on AgCl. The Nicolet MX-1 spectrometer was scanned 32 times (1 minute total) and the Fourier transform performed using triangular apodization. The major peaks at 1660 and 1547 cm^{-1} correspond to the amide I and II bands. The identification of other peaks in the spectrum is made in ref. 16. (b) The resonance Raman spectrum of purple membrane suspension measured with 5145 A excitation and a power of 30 mW. The sample was placed in a spinning cell rotating at 30 Hz which eliminated contributions from intermediates after L550. The Raman intensity has been displayed on an arbitrary negative scale for comparison to the FTIR difference spectrum. Only the protonated C=N appears. (c) The difference spectrum of 1a and a spectrum recorded immediately afterwards for one minute (32 scans) using illumination conditions described in the text. The frequency of some of the major peaks is shown. The spectrum was smoothed by a 17 point fit which resulted in 8 cm^{-1} resolution. However, the frequency of the peaks measured in different exeriments was repeatable to less than 1 cm^{-1}.

complete. This resulted in spectra of sufficiently high signal/noise ratio to obtain highly repeatable difference spectra. The difference between two one minute spectra recorded consecutively was used as a control to determine if the sample had reached equilibrium in the compartment.

RESULTS AND DISCUSSION: Figure 1a shows the spectrum of purple membrane film in the range 1400-1800 cm^{-1} recorded in the dark (results in other spectral regions will be described elsewhere). The assignment of peaks in this region along with a discussion of the dichroic origin of the relatively high ratio of the amide II to amide I peaks at 1660 and 1547 cm^{-1}, respectively, have been previously reported (11). The difference of the spectrum in Fig. 1a and a second spectrum recorded one minute later for an illuminated sample is shown in Fig. 1c. Although the maximum peak change in this spectrum is less than .003 OD, the spectrum is highly reproducible and

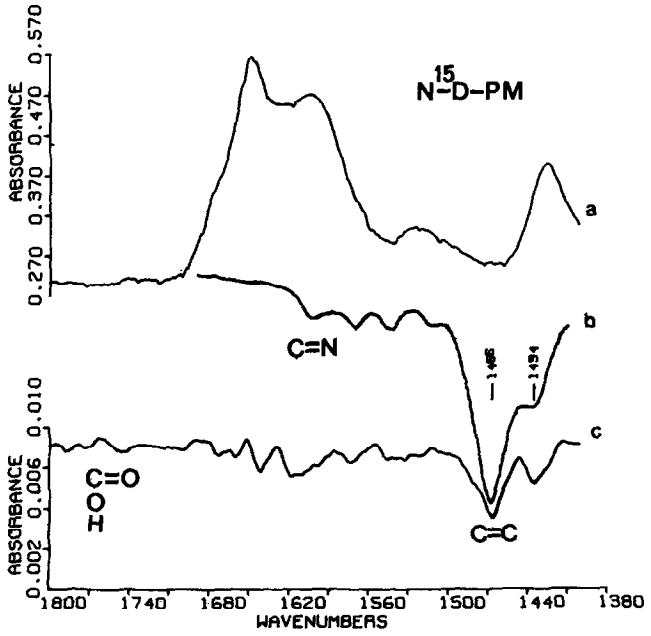

Figure 2. (a) Infrared absorption spectrum of purple membrane film containing a substituion of N^{15} for N^{14} and deuterium for hydrogen at all non-exchangeable positions. The labeled purple membrane was suspended in H_2O for 24 hours prior to film formation resulting in partial hydrogen-deuterium exchange at exchangeable groups such as peptide amides. (b) The resonance raman spectrum of the labeled purple membrane suspended in H_2O. The sample was contained in a capillary and the spectrum recorded using 5145 A excitation at 20 mW. The Raman intensity is displayed on a negative scale in order to compare with the FTIR difference spectrum. (c) Fourier transform difference spectrum of the labeled film using the same conditions as described in Figure 1c.

all of the labeled peaks were measured for several different films. In contrast, difference spectra of bleached purple membrane, where the chromophore is removed using hydroxylamine (20), revealed no such changes. After terminating the one minute illumination, subsequent one minute spectra revealed that the changes decay to a flat base line with a time constant of 4-5 minutes. Hence, the light induced FTIR differences correlate with the decay of the M412 intermediate.

Similar measurements (cf. Fig. 2a&c) were made on purple membrane films containing isotopic substitutions at all non-exchangeable positions of deuterium for hydrogen and N^{15} for N^{14} (N^{15}-D-PM, obtained courtesy of H. Crespi, National Argonne Laboratories). Assignments can be made on the basis of this data

and by comparison with resonance Raman spectra of normal purple membrane and N^{15}-D-PM (Fig. 1b and 2b). For example, the large negative peak at 1527 cm^{-1} in unlabeled purple membrane films (cf. Fig. 1c) agrees well with the frequency of the bR570 stretching vibration found at 1529 cm^{-1} in the resonance Raman spectrum (Fig. 1b) which reflects only vibrations of the chromophore (6-8). This assignment is strongly supported by the FTIR difference spectrum of N^{15}-D-PM film, where the largest negative peak is shifted to 1466 cm^{-1}, the same frequency as the C=C vibration of N^{15}-D-PM suspension measured by resonance Raman (Fig. 2b). The positive peak at 1566 cm^{-1} in Fig. 1c also correlates well with the frequency of the C=C stretching vibration of the M412 intermediate measured by resonance Raman (6-9). Hence, we conclude that the negative peak at 1530 cm^{-1} and the positive peak at 1567 cm^{-1} in Fig. 2b reflect the loss of bR570 and the production of M412 during illumination.

Several other points can also be made about the identification of the 1530 cm^{-1} peak. Since the C=C vibrations are the largest peaks observed in the resonance Raman spectrum their dominance in the IR difference spectrum indicates that, although the chromophore comprises less that 1% of the total weight of bacteriorhodopsin, its molecular alterations during the photocycle are large compared to other changes of the protein. The reduced size of the 1566 cm^{-1} peak relative to 1527 cm^{-1} is unexpected on the basis of the resonance Raman intensities and may be due to polarization effects. For example, this could occur if the C=C bond angles relative to the membrane plane increase in going from the all-trans form of the retinylidene chromophore in bR570 to the 13-cis form in M412. It should be possible to probe the orientation of these bonds by using polarized infrared spectroscopy. The shoulders observed near 1540 and 1550 cm^{-1} could also be related to the C=C vibrations of the L550 and X intermediates which exhibit similar peak frequencies in the resonance Raman spectrum. However, the absence of such shoulders in the N^{15}-D-PM film at a similar position relative to the 1476 cm^{-1} peak makes it more likely that these peaks are due to the amide II peptide vibrations of the protein.

We have also been able to demonstrate (21) that a negative band near 1646 cm^{-1} and a positive peak near 1620 cm^{-1} corresponding to the deprotonation of the Schiff base C=N vibration are present in the FTIR difference spectrum as well as the resonance Raman spectrum (cf. Fig. 2b and c). However, there are many peaks in the FTIR difference spectrum which cannot easily be accounted for by the vibrations of the retinylidene chromophore. In particular, there are a number of positive and negative peaks in the region from 1600-1800 cm^{-1} which do not match bands in the resonance Raman spectrum of bacteriorhodopsin. The positive peak at 1762 cm^{-1} is particularly interesting since it falls in a region where the carbonyl stretching vibration of COOH groups in aspartate and glutamate are found (22). It is unlikely that this vibration is from lipid ester carbonyl groups since this peak exhibits a 10 cm^{-1} shift to lower frequency in deuterated purple membrane film. This is characteristic of groups such as COOH containing an exchangable hydrogen (22). However, 1760 cm^{-1} is 10-15 cm^{-1} higher than that found in model compounds such as poly-benzyl-aspartate (23). One possible explanation is that the carboxylate group is perturbed by a nearby ionic charge thereby affecting its bond strength. Such a perturbation would be expected to also affect the pK_a of the group. In agreement, it has been found from model compound studies that a frequency of 1760 cm^{-1} corresponds to a pK_a of 2.5 or lower (24), 2 pH units lower than what is normally measured for the pK_a of aspartate and glutamate. Interestingly, a carboxylate group with a pK_a of 2.5 has been implicated in the photocycle on the basis of chemical labeling and the production of an acid 610 nm absorbing species (25,26).

This work demonstrates that FTIR difference spectroscopy can be used as a sensitive probe of light-induced conformational changes in bacteriorhodopsin. The advantages of this technique include the ability to determine the identity of groups undergoing changes as well as the possibility of measuring the orientational changes of these groups using polarized FTIR. Further progress might be made by using FTIR difference spectroscopy on bacteriorhodopsin containing specific isotope labels or chemical modification. In addition, it is possible to obtain time resolved

information by using FTIR kinetic methods (27) or flash induced kinetic infrared spectroscopy as recently applied to rhodopsin and bacteriorhodopsin (28). Finally, the sensitivity of this method indicates that it could be advantageously used in other biomembrane systems such as Ca^{++}-ATPase.

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