CONFORMATIONAL CHANGES IN BACTERIORHODOPSIN STUDIED BY INFRARED ATTENUATED TOTAL REFLECTION

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ABSTRACT We report on a new method based on Fourier transform infrared (FTIR)-difference spectroscopy for studying the conformational changes occurring during the photocycle of bacteriorhodopsin. Previous studies have been made by measuring the absorbance of an infrared (IR) beam transmitted through a thin hydrated purple membrane film. In contrast, the present study utilizes the technique of attenuated total reflection (ATR). Purple membrane is fixed on the surface of a germanium internal reflection crystal and immersed in a buffer whose pH and ionic composition can be varied. Measurements of the amide I and II absorbance with light polarized parallel and at 45° to the crystal surface reveals that the membrane is highly oriented. An ATR-FTIR-difference spectrum of the light to dark (bR_{570} to bR_{548}) transition is similar but not identical to the transmittance FTIR-difference spectrum. This disagreement between the two methods is shown to be due in the ATR case to the absorption of transition moments oriented predominantly out of the membrane plane. Raising the pH of La^{3+} substituted purple membrane films from 6.8 to 8.0 slows the M-decay rate sufficiently so that a bR_{570} to M_{412} difference spectrum can be obtained with steady state illumination at room temperature. A comparison of this difference spectrum with that obtained at −23°C using the transmittance method reveals several changes that cannot be attributed to out-of-plane transition moments. An increase in the intensity of peaks in the amide I and II regions agrees with recent time-resolved kinetic FTIR-difference measurements and indicates that a localized protein conformational change involving the peptide backbone of bR occurs which is not evident at the lower temperature.

INTRODUCTION

The membrane protein, bacteriorhodopsin (bR), functions as a light-driven proton pump in the purple membrane of *Halobacteria halobium* (1). While both the primary sequence (2) and the three-dimensional structure of bacteriorhodopsin at 7 Å have been reported (3), the molecular mechanism of proton pumping in bacteriorhodopsin still remains unknown.

Bacteriorhodopsin has been investigated recently by several groups using Fourier transform infrared (FTIR) difference spectroscopy (4-10; Roepe, P., P. L. Ahl, J. Herzfeld, S. K. Das Gupta, J. Lugtenburg, and K. J. Rothschild, manuscript to be submitted for publication). In contrast to resonance Raman spectroscopy, which selectively probes the vibrations of the bR chromophore (11, 12), FTIR-difference spectroscopy is sensitive to both protein and chromophore vibrations. Changes in chromophore, tyrosine, carboxyl, and peptide groups have all been detected at different steps in the bR photocycle using this method. Similar measurements have also been reported for the visual pigment rhodopsin (13) and for the bacterial photosynthetic reaction center complex (14).

One limitation of FTIR-difference spectroscopy stems from the strong infrared absorbance of water. This effect normally limits the sample thickness for transmittance measurements to less than a few micrometers. Typically, a thin film of purple membrane which has been deposited on an infrared transmitting window is hydrated by exposure to water vapor or placed in direct contact with an aqueous solution, which is then partially removed (4–7). While films of fully humidified purple membrane have been shown to photocycle with normal kinetics (15), it is difficult to control the water concentration, external pH, and ionic composition of these films. Furthermore, it has been demonstrated that the conformational changes occurring during the bR photocycle are strongly dependent on hydration level (15).

The limitation of FTIR absorption spectroscopy to samples with low water content can be circumvented through the use of attentuated total reflection (ATR). This method is based on absorption of the nonpropagating component of the infrared light (evanescent wave) which exists near the surface of a sample placed in direct contact

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with an internal reflection crystal (IRE). The infrared light is directed through the IRE at an angle that exceeds the critical angle for total internal reflection. In this case, infrared light penetrates several micrometers into the sample. Hence, the absorption due to the presence of a bulk aqueous solution is not a limiting factor (for a review of ATR theory and techniques see reference 16). When this method is combined with difference spectroscopy, small changes in the conformation of a sample in an aqueous environment can be detected. This approach has been employed recently by Fringeli et al. (17), who have studied the conformational changes of acetylcholine esterase upon changing the concentration of acetylcholine in the external solution. Changes in the conformation of bR as a function of the pH and ionic content of the buffer have also been recently studied using this method and will be reported elsewhere (17). (Marrero, H., and K. J. Rothschild, manuscript submitted for publication)

We report here on the first ATR FTIR-difference measurements of bacteriorhodopsin. Purple membrane was bound to the surface of a Ge crystal and directly exposed to a bulk aqueous solution whose composition and pH were varied. Difference spectra were recorded with the ATR method for the transition between light-adapted (bR_{570}) and dark-adapted (bR_{548}) bacteriorhodopsin and found to be similar but not identical to difference spectra recorded using the transmittance method. The differences between the two methods are shown to be due to out-of-plane vibrational modes of purple membrane which do not appear strongly in transmittance measurements. Polarized FTIR measurements also indicate that the purple membrane bound to the ATR crystal is highly oriented and provide information about the orientation of specific groups relative to the membrane plane. Raising the pH of the bathing solution above 8.0 for the La^{3+}-treated membranes slows the decay of the M_{412} intermediate sufficiently to allow $bR_{570} \rightarrow M_{412}$ difference spectra to be obtained at room temperature. These spectra are similar to the $bR_{570} \rightarrow M_{412}$ difference spectra recorded at 250°K using the transmittance technique (18), but more closely resemble recently measured time-resolved FTIR difference spectra of bR at room temperature (19).

MATERIALS AND METHODS

Purple membrane (PM) was prepared from *Halobacteria halobium* strain S9 as reported previously (5). Before binding of the PM, the Ge crystal was cleaned by immersion in concentrated chromic acid for 30 min, washed thoroughly in deionized water, and dried with either dry air or nitrogen. Care is taken to avoid contamination of the surface of the crystal. A thin layer of PM was deposited by drying a concentrated drop of PM suspension in distilled water (≈12 mg/ml) over the Ge crystal. The film was then extensively washed with either 65 mM $LaCl_3$ or 350 mM $CaCl_2$. The pH of the washing solutions was adjusted to pH 6.8 using NaOH for $LaCl_3$ or $Ca(OH)_2$ for $CaCl_2$. As discussed below, in the case of La^{+3} substitution, this method appears to produce effects on the bR photokinetics that are similar to those previously reported (20–22).

A schematic drawing of the apparatus used to measure the ATR difference spectra is shown in Fig 1. An ATR device (model TMP-220; Harrick Scientific Corp., Ossining, NY) equipped with a liquid cell

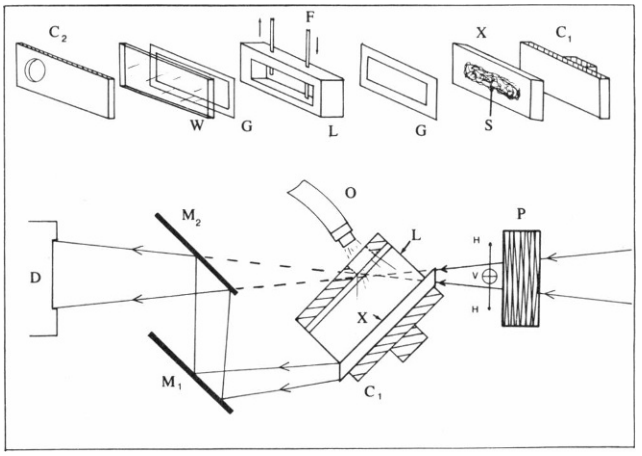

FIGURE 1 Cutaway schematic of modified ATR liquid cell including the liquid compartment assembly: clamp with light access hole (C_2), plastic or quartz window (W), rubber gaskets (G), liquid chamber made of Teflon (L), liquid input tubing (Teflon) (F), germanium ATR crystal (X), sample (S), and holding clamp (C_1). Bottom schematics show IR optical path including detector (D), mirrors (M_1, M_2), polarizer (P), and optical fiber (O).

allowed solutions to flow past the PM film bound to the surface of a Ge IRE. The sample was illuminated through a hole machined in the metal exterior of the cell (C_2 in Fig. 1) and covered by a plastic or quartz window. The light source consisted of a 100-W illuminator (model 180; Dolan-Jenner Industries, Inc., Woburn, MA). The light passed through two heat-absorbing filters and a yellow glass filter (No. 495; Corning Glass Co., Corning, NY) which was then directed with an optical fiber through the access hole. Control of the light source, polarizer, and all other FTIR spectrometer functions was automated using a computer (model 1280; Nicolet Instrument Corp., Madison, WI). Infrared spectra were measured using an FTIR spectrometer (model 60SX; Nicolet Instruments) equipped with either a TGS or MCT detector as indicated (cf. Fig. 4 legend). The path of infrared light through the ATR setup is shown in Fig. 1.

Polarized IR spectra were obtained by placing in the beam a KRS-5 wire-grid transmission polarizer mounted on a motorized rotator. By rotating the polarizer 90°, two different polarizations were obtained (cf. Fig. 1); the IR electric vector parallel to the surface of the crystal (VP mode) and the IR electric vector at 45° with respect to the normal of the crystal (HP mode).

In order to test the stability of the PM binding to the Ge crystal when it is exposed to aqueous solutions, IR spectra were recorded over several hours. Even when the solution was rapidly flowing through the liquid chamber no evidence was found of PM loss from the surface of the crystal.

In order to study the bR photokinetics, PM was bound to the inside surface of a plastic or quartz cuvette using the same fixing procedure described above. Visible absorbance measurements were made in a double beam UV-visible spectrometer (model 219; Varian Associates, Inc., Palo Alto, CA) using the conventional tramsmittance method.

RESULTS AND DISCUSSION

Effects of PM Binding on the Photochemical Behavior of Bacteriorhodopsin

Fig. 2, *A–C* compare the visible absorption spectrum of light- and dark-adapted purple membrane in suspension, for films treated with 350 mM $CaCl_2$ and 65 mM $LaCl_3$,

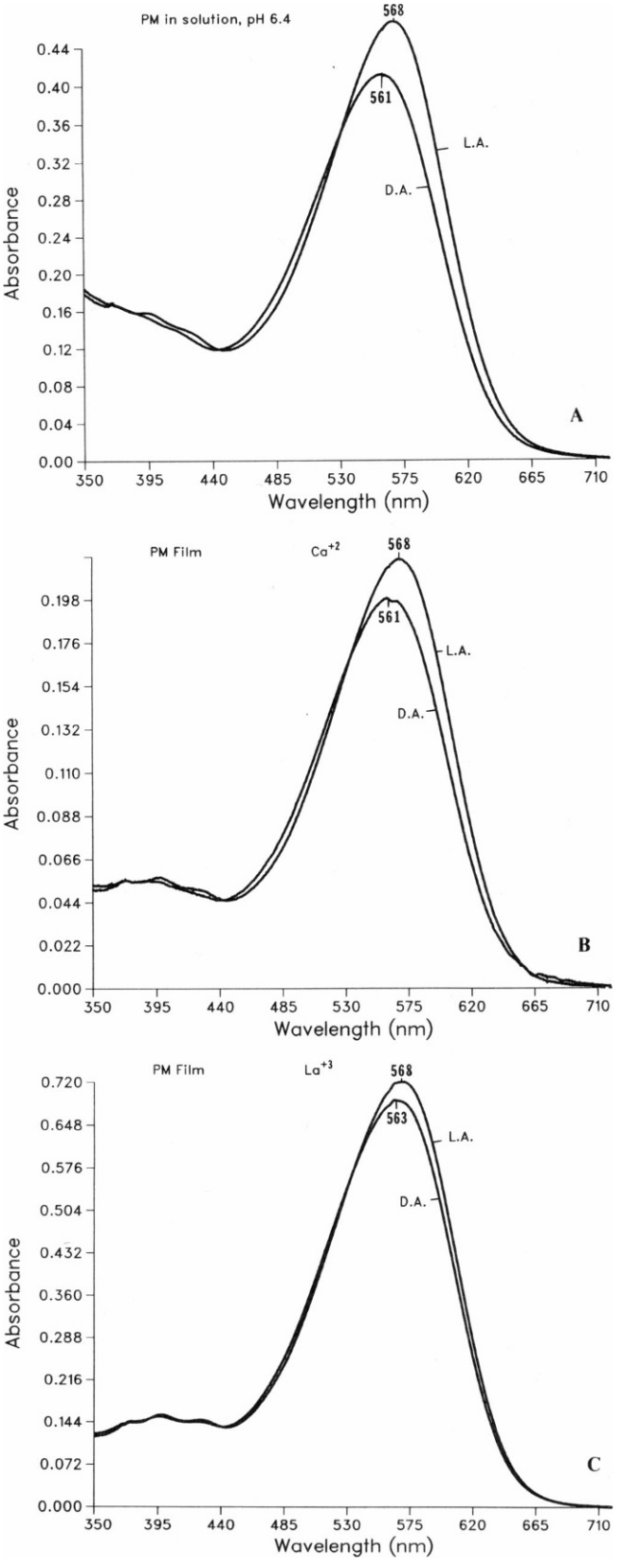

FIGURE 2 Visible absorbance spectra for PM. (*A*) PM suspension in distilled water at pH 6.4, (*B*) PM film fixed inside a quartz cuvette with a 0.35 M $CaCl_2$ solution at pH 6.8, and (*C*) PM film fixed in a quartz cuvette in a solution at pH 6.8 and 0.065 M $LaCl_3$. The samples were dark adapted for 2.5 h before light adaptation.

respectively. Both the $CaCl_2$- and the $LaCl_3$-treated films exhibit normal light adapted spectra and the expected blue shift upon dark adaptation. However, La^{3+}-substituted PM does not exhibit as great a reduction in absorbance as normally observed upon dark adaptation for PM in suspensions (23 and references therein) and for PM treated with Ca^{2+} as shown here. This effect may be due to incomplete dark adaptation of the La^{3+}-treated samples. The rate of dark adaptation was found to be much slower for both kinds of films (Ca^{2+}- and La^{3+}-treated films); more than 2 h were required as compared with ~20–30 min for PM in suspension (results obtained here; see also references in 23). Hence, longer dark-adaptation times were allowed for the FTIR measurements described below. It was also found that the M_{412} decay was much slowed in the $LaCl_3$-treated films above pH 7.6 (having a $t_{1/2}$ of 1–5 s at pH 8.0), in agreement with studies made for La^{3+}-treated purple membrane fixed in acrylamide gels (20–22).

Orientation of Purple Membrane

The orientation of the purple membrane relative to the IRE crystal surface can be determined from polarized infrared ATR measurements. As shown in Fig. 3, the amide I/amide II ratio for the in-plane VP polarization is <1, whereas the ratio increases to ~1.75 for the out-of-plane HP polarization. Both of these effects, although less pronounced, have been previously observed for polarized transmittance measurements of purple membrane films (24) and have been attributed to the predominantly perpendicular orientation of bR α-helices relative to both the membrane and sample planes. In the present case, we would not expect to observe such high amide I and II

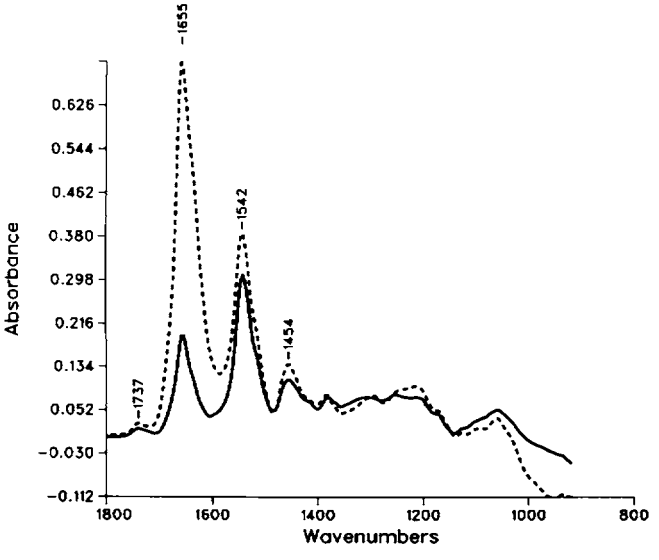

FIGURE 3 ATR-FTIR absorbance spectra of a $LaCl_3$-treated film deposited on Ge in the presence of 0.065 M $LaCl_3$ (pH 6.8). The spectra were calculated using as a background an identical ATR cell without purple membrane. Dashed line (horizontal polarization); solid line (vertical polarization).

dichroism if the PM membranes were not highly oriented such that the membrane plane is parallel to the Ge surface. This is not surprising, since the fixing method involves the initial drying of PM suspensions, which normally leads to a parallel orientation of the fragments relative to a substrate surface. Furthermore, it has been shown that the maximum orientation possible is limited by edge overlap of the fragments, the extent of disorder increasing as a function of the distance from the substrate surface (25). Therefore, the relatively thin layer of purple membrane bound, which is estimated on the basis of optical fringing to be between 0.5 and 1.5 μm, would tend to produce higher orientation than the 3 μm films previously used for transmittance measurements.

Light → Dark Adaptation Difference Spectra

Fig. 4 shows the light-dark difference spectra ($bR_{570} \rightarrow bR_{548}$) obtained using the ATR and transmittance methods. We first note that all of these spectra are in qualitative agreement with previous FTIR transmittance measurements (6). Chromophore vibrations previously detected by resonance Raman spectroscopy of bR_{570} and bR_{548} (29) appear as negative peaks (bR_{570}) and as positive peaks (bR_{548}). For example, the two strong peaks at 1,525 cm^{-1} (negative) and 1,535 cm^{-1} (positive) in the FTIR difference spectrum arise from the ethylenic stretching modes of the chromophore in the light- and dark-adapted forms of bR, respectively. Similarly, the negative 1,637 cm^{-1} peak is assigned to the bR_{570} C=N stretch mode of the Schiff base. Several protein-related peaks can also be identified, including the small feature at 1,276 cm^{-1} which has been attributed to the protonation of a tyrosine group during the $bR_{570} \rightarrow bR_{548}$ (9–10; Roepe P., P. L. Ahl, J. Herzfeld, S. K. Das Gupta, J. Lugtenburg, and K. J. Rothschild, manuscript submitted for publication).

While the ATR difference spectra for both bR containing Ca^{2+} and La^{3+} at pH 6.8 appear to qualitatively agree with the transmittance difference spectrum, there exist several reproducible differences between the two methods. These include intensification of the peak at 1,660 cm^{-1}, loss of intensity at 1,621 cm^{-1}, and the appearance of a negative feature near 1,615 cm^{-1} in the ATR difference spectra.

In principle, all of the above noted changes in the ATR difference spectra could arise from the contribution of vibrational modes with transition dipole moments containing a component oriented out of the membrane plane. In particular, as shown in Fig. 1, the infrared light propagating through the IRE is oriented at 45° relative to crystal surface and therefore to the membrane plane. Hence, the component of the electric field normal to the membrane that exists for the HP but not for the VP polarization will interact more with transition moments oriented out-of-plane. In contrast, in the case of transmittance measurements, the light is directed normal to the membrane plane and hence out-of-plane transitions are not excited as strongly.

This effect was verified by recording the ATR difference spectra with infrared light polarized parallel (VP) and perpendicular (HP) to the crystal surface (cf. Fig. 1). The VP (in-plane) polarization results in a difference spectrum (thick line in Fig. 5) very similar to the transmittance difference spectrum. In contrast, the HP (out-of-plane) polarization results in a spectrum (thin line in Fig. 5) which deviates from the transmittance difference spectrum at the same frequencies as the unpolarized ATR

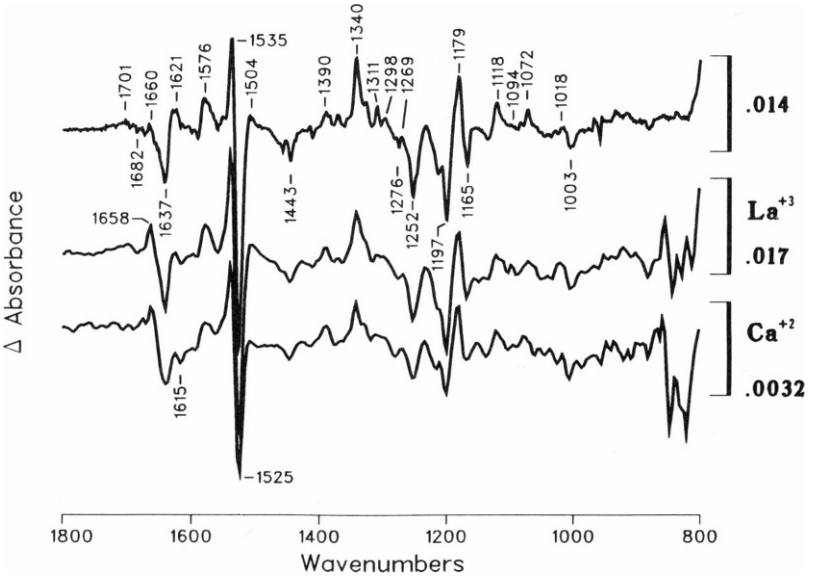

FIGURE 4 Comparison of FTIR-difference spectra of the bR_{570} to bR_{548} transition (dark adaptation) for different methods. (*Top*) Transmittance method obtained at room-temperature on a humidified PM film deposited on AgCl. (*Middle*) ATR method obtained at room-temperature on a PM film deposited on Ge and bathed by a 0.065-M $LaCl_3$ solution at pH 6.8. (*Bottom*) Same as *middle* but with a solution containing 0.35 M $CaCl_2$ at pH 6.8. The numbers at the right of each spectrum indicate the respective absorbance scale. All ATR spectra shown were obtained at an effective resolution of 8 cm^{-1} using a model 60SX spectrometer (Nicolet Instruments) equipped with an MCT detector. Data collection consisted of 2,048 data points for each interferogram, with 10,000 interferograms recorded for each spectrum shown, for a total data acquisition time of 41 min. The sample was allowed to dark-adapt for 2 h before illumination. The difference spectrum was computed by subtracting the absorption spectrum of the sample during illumination from the spectrum recorded in the dark immediately before illumination. The top difference spectrum was recorded at 2 cm^{-1} resolution at room temperature using a model MX-1 spectrometer (Nicolet Instruments) equipped with a TGS detector.

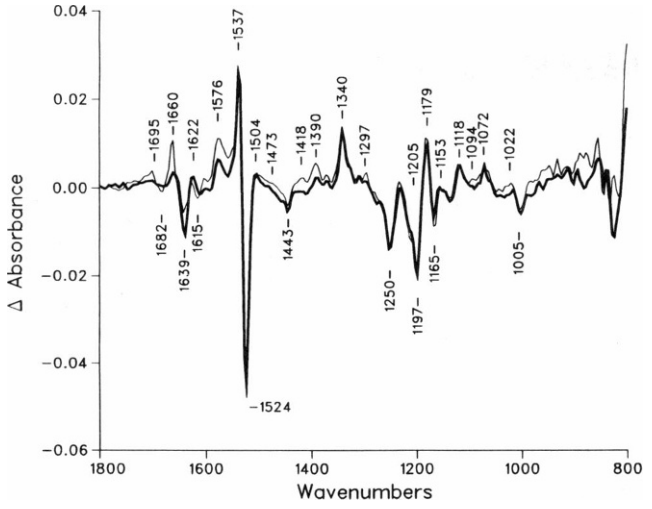

FIGURE 5 Polarized ATR FTIR-difference spectrum for a film bathed in a pH 6.8 solution of 0.065 M $LaCl_3$ at room temperature using conditions identical to those in Fig. 4. Difference spectra of the same polarization were averaged together for increased signal/noise. *Thick line*, VP polarization; *thin line*, HP polarization (see Materials and Methods).

difference spectrum, but to a greater extent. This demonstrates that it is the predominantly out-of-plane transition moments not detected in the transmittance measurements which account primarily for the disagreement between the transmittance and ATR measurements.

We also note that polarized ATR-FTIR difference measurements can provide information about the orientation of specific transition moments relative to the membrane plane. Such studies have been recently reported using transmitted IR for the bR_{570} to K_{630}, and M_{412} transitions (26, 27). We list in Table I the predominant polarization behavior of specific peaks in the spectrum. A more detailed analysis of the dipole orientations will be given elsewhere.

BR_{570} to M_{412} Differences for La^{3+}-PM at pH 8.0

The difference spectrum of La^{3+}-treated PM at pH 6.4 (cf. Fig. 4) reflects mainly light-dark adaptation. However, raising the pH to 8.0 results in a dramatically altered spectrum (cf. Fig. 6). In particular, at pH 8.0 the difference spectrum is very similar (although not identical) to previously reported bR_{570} to M_{412} difference spectra recorded at low temperature (6, 8, 10, 18). This effect can be explained by the strong pH-dependence of the M_{412} decay kinetics of La^{3+}-PM. We found the M_{412} decay to be several hundred times slower at pH = 8 compared with pH = 6.4 (Fig. 7), in agreement with previous studies using La^{3+}-PM in acrylamide gels (20–22). Thus, the M_{412} intermediate accumulates during the illumination period and relaxes back to bR_{570} during the dark periods.

Some of the differences between the $bR_{570} \rightarrow M_{412}$ spectra shown in Fig. 6 for the transmittance and ATR methods may be again due to out-of-plane modes appearing more strongly in the ATR case. For example, the increased intensity of the 1,648 cm^{-1} peak agrees with the out-of-plane character of this peak as recently demonstrated (27). However, some changes such as the increase at 1,553 cm^{-1} cannot be fully explained on this basis and may be due in general to either the presence of La^{+3}, the presence of bulk water, or the room temperature conditions. Recent time-resolved measurements made on room temperature wet samples in our laboratory (19) support the latter explanation. Furthermore, polarized FTIR difference measurements indicate that this peak is not polarized out-of-plane (27).

TABLE I

Frequency	Type*	Dichroism	Assignment‡
cm^{-1}			
1,695	(+)	HP > VP	Protein
1,682	(−)	HP < VP	Protein
1,660	(+)	HP > VP	Protein, C═O stretch
1,639	(−)	VP < HP	C═N^+ stretch
1,622	(+)	VP > HP	
1,615	(−)	HP < VP	
1,576	(+)	HP > VP	C═C stretch
1,537	(+)		C═C stretch
1,525	(−)		C═C stretch
1,504	(+)		
1,418	(+)	HP > VP	
1,390	(+)	HP > VP	H in-plane rock
1,340	(+)		H in-plane rock
1,297	(+)	HP > VP	H in-plane rock
1,268	(+)		tyrosine
1,250	(−)		C_{12}-C_{13} stretch and lysine rock
1,205	(+)	VP > HP	
1,197	(−)		C_8-C_9, C_{14}-C_{15} stretch
1,179	(+)	HP > VP	C-C stretch
1,165	(−)	HP < VP	C_{10}-C_{11} stretch
1,118	(+)		
1,094	(+)	HP > VP	
1,067	(+)	VP > HP	C-C stretch
1,022	(+)	HP > VP	
1,005	(−)		CH_3 rock of C_{19} and C_{20}

*(+), positive amplitude (dark, bR_{548}); (−), negative amplitude (light, bR_{570}); VP, polarization parallel to crystal plane; HP, polarization at 45° from crystal normal.

‡Chromophore assignments based on assignments in references 28 and 29.

CONCLUSIONS

The present study demonstrates that ATR FTIR-difference spectroscopy can be used to study conformational changes occurring in purple membrane. Since the membranes are directly exposed to an aqueous medium, the pH and ionic content of the buffer can be controlled during an experiment. Thus, the effects of the buffer composition on the conformational changes of bR during the photocycle can be more easily investigated in contrast to previous experiments where hydrated thin films of purple membrane were utilized.

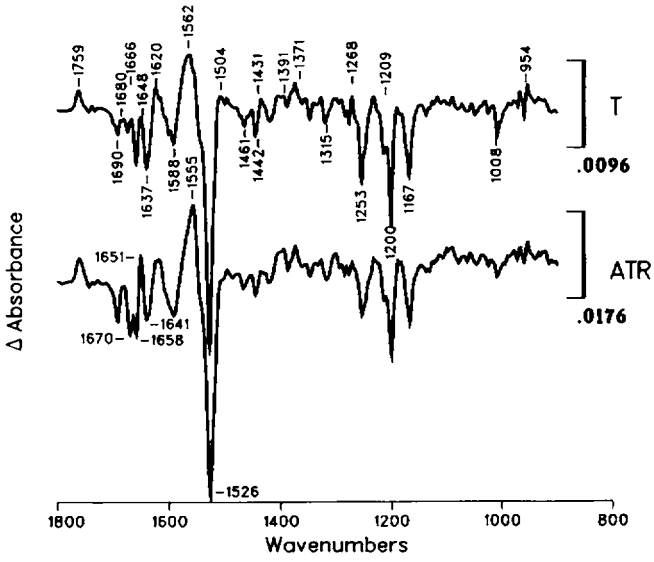

FIGURE 6 Comparison of FTIR-difference spectra of the bR_{570} to M_{412} transition. (*Top*) Difference spectrum obtained at −23°C using the transmittance method as previously reported (from reference 18). (*Bottom*) Difference spectrum obtained at room temperature using the ATR method. Bathing solution consists of 0.065 M $LaCl_3$ and was raised from pH 6.8 (conditions used for dark adaptation) to pH 8.0 using NaOH in order to obtain spectra shown here. pH was continuously monitored during measurement by sampling solution with a syringe mounted to ATR cell as shown in Fig. 1. Spectra were collected at 3-min intervals (750 interferograms per spectrum at 8 cm^{-1} resolution), with alternating light-on/light-off.

These studies also demonstrate the capability of polarized ATR FTIR-difference spectroscopy to probe the orientation of different groups which change during the bR photocycle. Similar studies using the transmittance method of FTIR-difference spectroscopy have also been recently reported (26, 27). In the case of ATR, the ability to obtain a larger effective angle between the electric polarization and the membrane plane as well as the ability to maintain a highly oriented film in an aqueous medium offers important advantages.

One disadvantage of the present approach is the necessity of using high salt concentrations to fix the films to the IRE surface. Without the salt, we found the films to be unstable during the course of the experiment, resulting in unsatisfactory difference spectra. This problem may be avoidable in future experiments by finding alternative methods of binding purple membrane to the IRE surface, such as through the use of surfactants or with a polyacrylamide matrix. Alternatively, concentrated suspensions of PM may be used directly in an appropriate ATR cell, without the need for film deposition.

The ability to vary the external pH of the bathing medium has allowed us in this work to effectively control the M_{412} decay rate in La^{3+}-PM films. Our comparison of the resulting $bR_{570} \rightarrow M_{412}$ difference spectrum with previously recorded low-temperature measurements indicates that skeletal backbone rearrangements occur which are not as evident in 250°K difference spectra. This is supported by recent time-resolved FTIR difference measurements at room temperature of normal PM films which also exhibit intensification of bands associated with the amide I and II vibrations (19), as well as static difference spectra recorded at 270°K (Roepe et al., unpublished observations).

The ATR FTIR-difference method can also be used to study changes in bR when the pH and ionic concentrations are varied. For example, we have used this method to study conformational changes in bR upon acid blue membrane formation in the presence of different salt concentrations (Marrero, H., and K. J. Rothschild, manuscript submitted for publication). In general, further development of this method may also facilitate the study of a wider class of proteins which are activated by soluble substrates and not just light activation as in the case of bacteriorhodopsin.

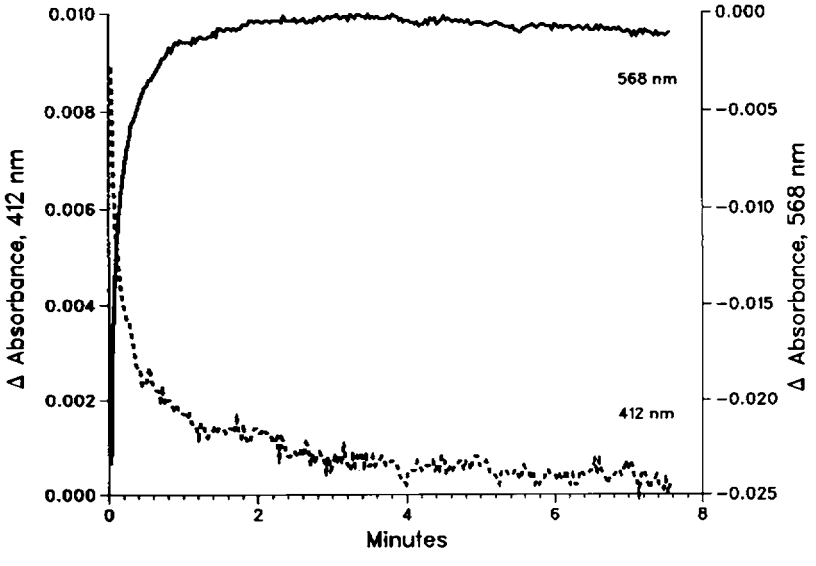

FIGURE 7 Kinetic decay of the 412 nm (M_{412} intermediate) and 570 nm (bR_{570}) bands recorded immediately after the light source was turned off for a sample fixed inside a quartz cuvette in the presence of 0.065 M $LaCl_3$ at pH 8.0. The sample was light adapted in the same manner as the samples described in Fig. 2. Left absorbance scale refers to change at 412 nm and right absorbance scale refers to change at 568 nm after light is turned off relative to final value.

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