Polarized Fourier transform infrared spectroscopy of bacteriorhodopsin

Transmembrane alpha helices are resistant to hydrogen/deuterium exchange

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ABSTRACT The secondary structure of bacteriorhodopsin has been investigated by polarized Fourier transform infrared spectroscopy combined with hydrogen/deuterium exchange, isotope labeling and resolution enhancement methods. Oriented films of purple membrane were measured at low temperature after exposure to H_2O or D_2O. Resolution enhancement techniques and isotopic labeling of the Schiff base were used to assign peaks in the amide I region of the spectrum. α-helical structure, which exhibits strong infrared dichroism, undergoes little H/D exchange, even after 48 h of D_2O exposure. In contrast, non-α-helical structure, which exhibits little dichroism, undergoes rapid H/D exchange. A band at 1,640 cm^{-1}, which has previously been assigned to β-sheet structure, is found to be due in part to the $C = N$ stretching vibration of protonated Schiff base of the retinylidene chromophore. We conclude that the membrane spanning regions of bR consist predominantly of α-helical structure whereas most β-type structure is located in surface regions directly accessible to water.

INTRODUCTION

Bacteriorhodopsin (bR) is a light-driven, transmembrane proton pump located in the purple membrane (PM) of *Halobacterium halobium*. Its function is to vectorially translocate protons from the cell's interior to the external medium, thereby creating an electrochemical potential which is coupled to the synthesis of ATP (Stoeckenius and Bogomolni, 1982). The three-dimensional structure of bR determined by electron diffraction and electron microscopy shows seven α-helical segments which are oriented predominantly perpendicular to the membrane plane (Henderson and Unwin, 1975; Hayward and Stroud, 1981; Henderson et al., 1986, 1990; Tsygannik and Baldwin, 1987). However, electron density projections in the membrane plane have also been interpreted by Jap et al. (1983) as consistent with five α-helical segments and four β-strands which are oriented perpendicular to the membrane plane.

Several spectroscopic techniques detect β-type structure in bR, although there is disagreement about the amount. UV-CD and fluorescence UV-CD (Jap et al., 1983) detect 50% α-helix and 20% β-sheet content which is consistent with the existence of several transmembrane β-strands. However, two other UV-CD studies of bR detect less β-structure (Nabedryk et al., 1985; Wallace and Teeters, 1987). A Raman study of the secondary structure of the apoprotein, bacterio-opsin (bO), determined the α-helical content to be 72–82%, with 2–11% β-sheet and 11–17% β-turn (Vogel and Gärtner, 1987).

Several infrared studies also support the existence of β-structure in bacteriorhodopsin (Jap et al., 1983; Lee et al., 1985, 1987; Earnest and Rothschild, 1986; Downer et al., 1986). This evidence is based largely on the existence of amide I bands ($C=O$ stretching mode) at frequencies characteristic of β-sheet structure (Fraser and MacRae, 1973). However, there is not very close agreement between the reported frequencies of these bands in the PM infrared spectra. Furthermore, it is difficult to compare these different studies because they were made under a variety of conditions with variable water content and degree of purple membrane orientation relative to the probing IR beam. This latter factor can alter the amide I band contour due to changes in the distribution of the amide I transition moments relative to the sample normal in an uniaxially oriented multilamellar array (Rothschild and Clark, 1979; Nabedryk et al., 1985).

None of the previous spectroscopic studies were able to determine whether β-type structure is part of the membrane spanning regions of the protein. In the present work, we have measured the infrared absorption and linear dichroism of purple membrane films exposed to H_2O or D_2O. Low-temperature and Fourier self-deconvolution methods were used to separate the individual components of the amide I band. Using isotope labeling of lysines, we demonstrate that a band previously assigned to β-type structure is due in part to the Schiff base $C = N$

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stretch mode. A well-oriented α-helical "core" structure of bR is found which is very resistant to D/H exchange. In contrast, β-type structure is detected which undergoes rapid D/H exchange. Our results are consistent with the standard model of bR which envisions a core of transmembrane buried α-helices and a surface region which contains some β-type structure. Significantly, these infrared based techniques can also be applied to other membrane proteins for which the structure is unknown.

METHODS

bR and ^{15}N-lys-bR were grown and isolated as described in Argade et al. (1981). Oriented multilamellar films of PM on AgCl windows were formed by the isopotential spin-dry (ISD) method (Clark et al., 1980). The individual PM fragments in such films have been shown previously by electron microscopy to be oriented parallel to the sample plane with a small mosaic spread which is due mainly to edge overlap of the fragments. Linear dichroism measurements at 570 nm of samples oriented by this method on glass coverslips indicate the films are rotationally isotropic about the sample normal. The chromophore transition moment is tilted at ~67° ± 2° to the sample normal (Clark et al., 1980).

Polarized Fourier transform infrared (FTIR) measurements at 80 ± 0.1 K and 250 ± 0.1 K were made using a 60SX spectrometer (Nicolet Analytical Instruments, Madison, WI) equipped with a liquid N_2-cooled HgCdTe detector using methods described previously in Earnest et al. (1986). All spectra were recorded by accumulating a minimum of 2,000 interferograms scans. The averaged interferograms were apodized using a modified Happ-Genzel function and Fourier-transformed. The effective spectral resolution was 2 cm^{-1}. The AgCl window with the deposited sample was loaded into a homebuilt cell containing a second AgCl window and an O-ring spacer. The two windows are compressed against the O-ring by screws in the cell housing to obtain sealing. Hydration of the film was accomplished by applying a drop of water to the film and then removing excess water by shaking or evaporation. Deuteration of bacteriorhodopsin was performed by exposing a dehydrated film of purple membrane to D_2O for various periods of time. Before the FTIR measurement, the sample was immediately inserted into a sealed transmittance cell and cooled to 80 K. Analysis of the linear dichroism was performed as outlined in Earnest et al. (1986) and Earnest (1987).

RESULTS

Fig. 1 shows the FTIR spectra of a hydrated film of purple membrane in the amide I and II region for $A_\parallel$ (*solid*) and $A_\perp$ (*dashed*) along with a plot of the linear infrared dichroism defined as $A_\parallel - A_\perp$. The dichroism plot exhibits a positive peak at 1,667 cm^{-1} and negative peak at 1,547 cm^{-1} reflecting the net orientation of the bR α-helices perpendicular to the membrane plane (Rothschild and Clark, 1979). These results are in close agreement with previous measurements made on dehydrated purple membrane (Rothschild and Clark, 1979; Nabedryk et al., 1985) and give an estimated average α-helix tilt angle of 24–27° (Earnest, 1987).

The shoulders near 1,685 and 1,640 cm^{-1} in Fig. 1 are

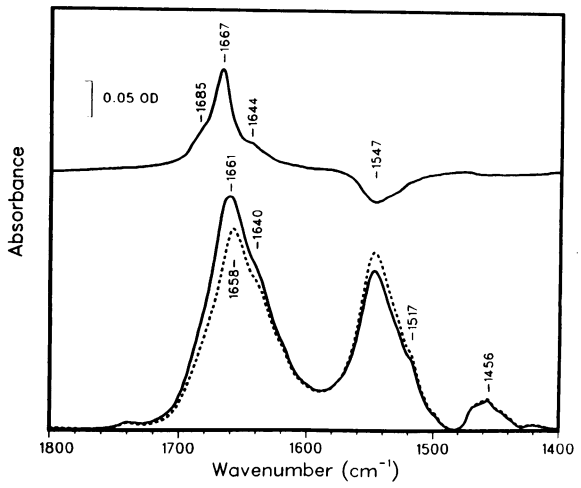


FIGURE 1 Polarized FTIR spectra of hydrated purple membrane at 280 K. (*Bottom*) Spectra taken with incident beam polarized perpendicular, $A_\perp$, (*dashed line*) and parallel, $A_\parallel$, (*solid line*) to the plane of incidence. (*Top*) $A_\parallel - A_\perp$.

of special interest because they may be due to β-sheet structure in the antiparallel or parallel conformation (Fraser and MacRae, 1973). Alternatively, β-turns can produce amide I bands above 1,670 cm^{-1} region and also in the 1,620–1,640 cm^{-1} region (Bandekar and Krimm, 1979).

To probe the accessibility of different bR structural components to aqueous media, we examined changes in the IR absorption and linear dichroism after PM exposure to D_2O for 2 and 48 h. As seen in Fig. 2, the major effect of D/H exchange after 2 h is to lower the overall absorption in a region around 1,685 and 1,547 cm^{-1} while increasing the relative intensity near 1,460 cm^{-1}, in agreement with the same pattern seen in the resolution enhanced spectra. In addition, the region near 1,639 cm^{-1} decreases in intensity while the region near 1,631 cm^{-1} gains intensity.

This effect can also be seen in Fig. 3, which compares the low-temperature spectra of the hydrated and the deuterated samples for both orientations of polarized light at 2 and 48 h after exposure to D_2O. The curve shown at the bottom is a difference spectrum between bR in H_2O and in D_2O after 2 h. D_2O exposure results in the downshift of bands at 1,684 and 1,640 cm^{-1} to 1,658 and 1,627 cm^{-1}, respectively. The downshift of the 1,640 cm^{-1} band accounts for the appearance of a pronounced shoulder near this frequency in the D_2O spectra.

The total extent of D/H exchange of the bR peptide groups can be determined approximately from the downshift of the amide II mode from the 1,545 cm^{-1} region to near 1,460 cm^{-1}. The ratio of the amide II′ to amide I′ band intensities, ω_D, in these spectra is related to f_u the

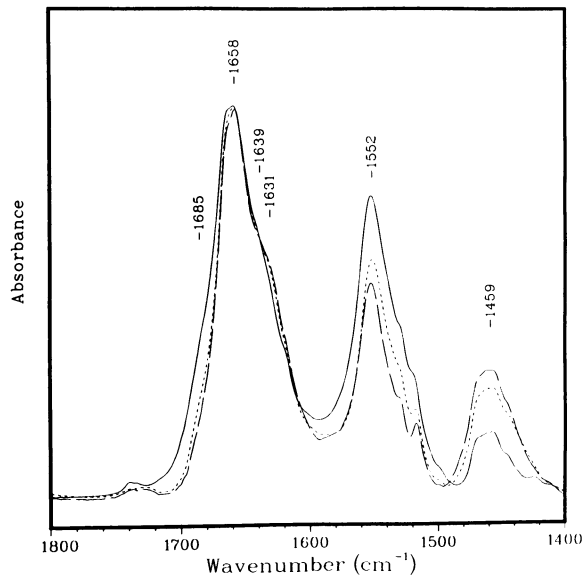


FIGURE 2 Spectra of PM: hydrated (*solid*), exposure to D_2O for 2 h (*short dashed line*), and exposure to D_2O for 48 h (*long dashed line*). Recorded at 250 K with incident polarization parallel to the plane of incidence.

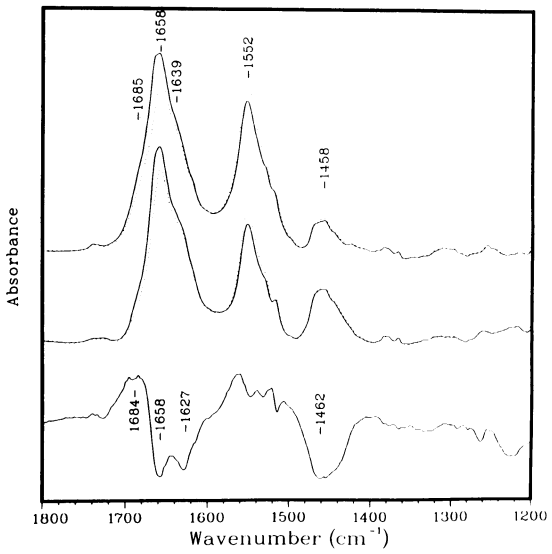


FIGURE 3 Polarized FTIR spectra of hydrated PM (*top*) and PM after 48 h of exposure to D_2O (*middle*) taken with incident polarization parallel (*solid*) and perpendicular (*dotted*) to the plane of incidence. Bottom curve is rescaled difference spectra of hydrated minus deuterated samples.

fraction of amide groups which remain unexchanged by:

$$f_u = \omega_D \omega_H,$$

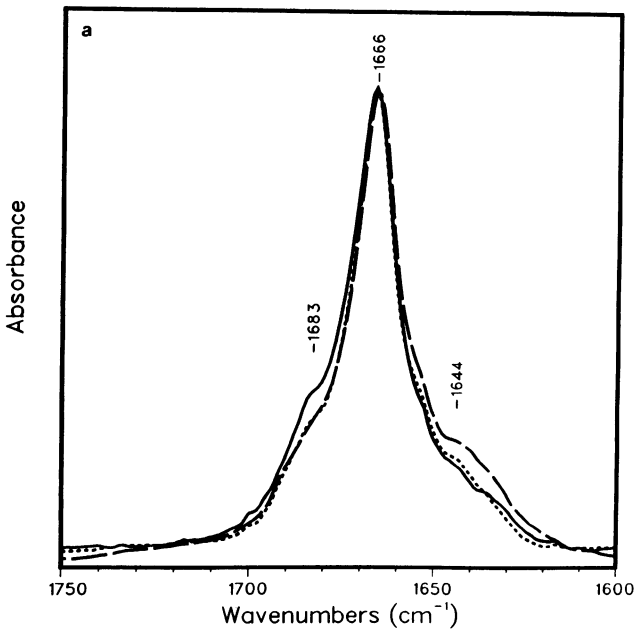
where ω_H is the amide II/amide II ratio before D/H exchange in a dehydrated sample. While this estimate is prone to error due to changes in the amide II band shape and possible nonamide II contributions near 1,545 cm^{-1} and 1,460 cm^{-1}, including possible contributions from HOD, calculations indicate that approximately 80% of the peptide groups are unexchanged after 2 h and 73% after 48 h.

In contrast to the marked changes observed in the infrared spectrum of PM due to D_2O exposure, the dichroism spectra show little effects of D/H exchange. Only a small increase in a negative peak is found near 1,460 cm^{-1} in the dichroism spectra of the D_2O samples compared with the H_2O exposed sample (Fig. 4). *Thus, while significant D/H exchange occurs in bR peptide groups after only 2 h, it involves almost exclusively nondichroic secondary structure which has little net orientation.* The small amount of D_2O sensitive linear dichroism detected in the amide I region occurs at ~1,683 cm^{-1} and 1,640 cm^{-1} (Fig. 4 *a*).

To examine in greater detail the changes occurring in the amide I bands upon D/H exchange, we utilized the resolution enhancement method of Fourier self-deconvolution (Kauppinen et al., 1981; Earnest et al., 1988). Fig. 5 shows the deconvolved spectra of PM at 250 and 80 K. Both spectra exhibit very similar fine structure, although as expected, the bands in the low temperature spectrum are narrower. Most notable in the resolution enhanced spectra is the splitting of the 1,685 and 1,640 cm^{-1} shoulders into five subbands, located at 1,685, 1,676, 1,639, 1,631, and 1,618 cm^{-1}. These bands were found to be present in all bR samples examined both by this method and second derivative resolution enhancement (Earnest, 1987). The main amide I peak in the 1,660 cm^{-1} region is also split into perpendicular and parallel components as previously found (Rothschild and Clark, 1979).

Fig. 6 compares the resolution enhanced spectra of PM in H_2O and D_2O at 80 K. One prominent effect of D_2O is the downshift of intensity of the 1,639 cm^{-1} band to 1,629 cm^{-1}. This band has been previously assigned to β-type structure (Downer et al., 1986; Lee et al., 1985, 1987), but may also arise from the $C{=}N$ stretch mode of the protonated Schiff base in bR_{570} which has a frequency at 1,640 cm^{-1} and shifts to 1,627 cm^{-1} upon D/H exchange.

To check this possibility, we measured the effect of photoconversion of bR_{570} to K_{630} on this band, which should shift to 1,609 cm^{-1} if it arises from the $C{=}N$ stretch mode (Rothschild et al., 1984). As shown in Fig. 7, a reduction in intensity at 1,639 cm^{-1} band and an increase in intensity is observed at 1,609 cm^{-1}. Furthermore, the resolution enhanced spectrum of bR ^{15}N-ϵ-lysine shows an increase absorption in 1,630 cm^{-1} and reduction in intensity at 1,640 cm^{-1} corresponding ex-



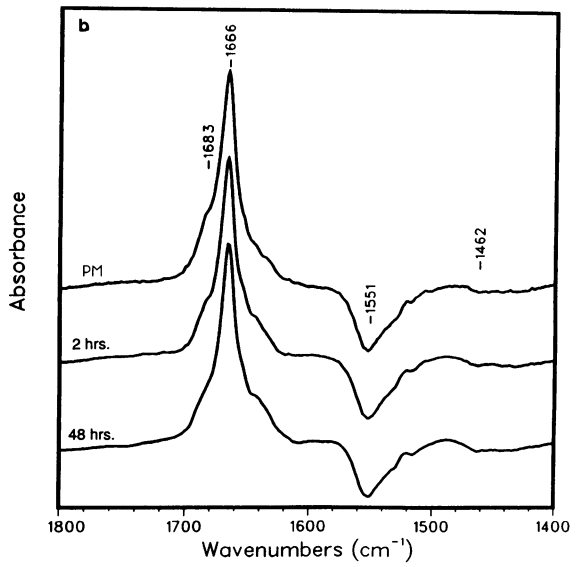


FIGURE 4 (*a*) $A_{\parallel} - A_{\perp}$ for hydrated PM (*top*), PM after 2 h of exposure to D_2O (*middle*) and PM after 48 h of exposure to D_2O. (*b*) Overlay of figures from top (*solid*), middle (*short dashed*), and bottom (*long dashed*) over the 1,750–1,600 cm^{-1} region.

actly to the expected isotope induced shift of the $C{=}N$ mode due to ^{15}N labeling. Photoconverting of the ^{15}N-lysine labeled bR from bR_{570} to K_{630} causes a reduction in intensity at 1,630 cm^{-1} instead of 1,640 cm^{-1}. Because the fraction of bR_{570} converted to K_{630} is ~30% under optimal conditions (Hurley and Ebrey, 1978) at least

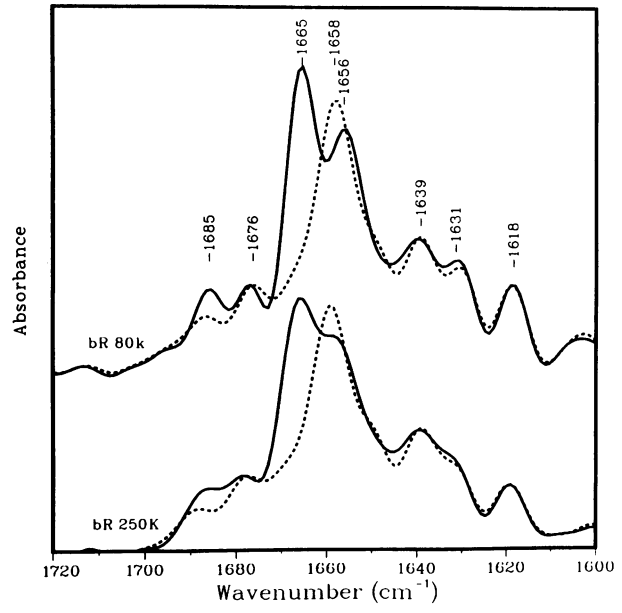


FIGURE 5 Amide I region for hydrated PM at 80 K (*top*) and 250 K (*bottom*) with parallel (*solid*) and perpendicular (*dashed*) polarization after Fourier self-deconvolution (half width = 16 cm^{-1}, k = 2.0).

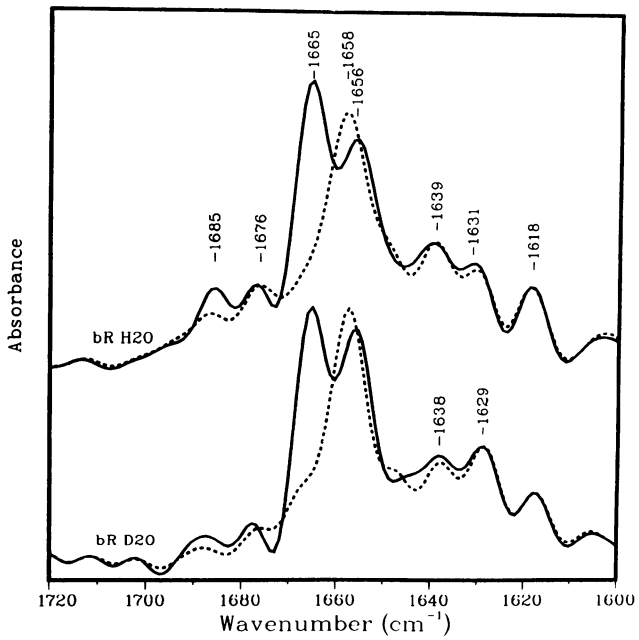


FIGURE 6 Amide I region for hydrated (*A*) and deuterated after 48 h (*B*) PM recorded at 80 K with parallel (*solid*) and perpendicular (*dashed*) polarization. Deconvolution parameters are as in Fig. 5.

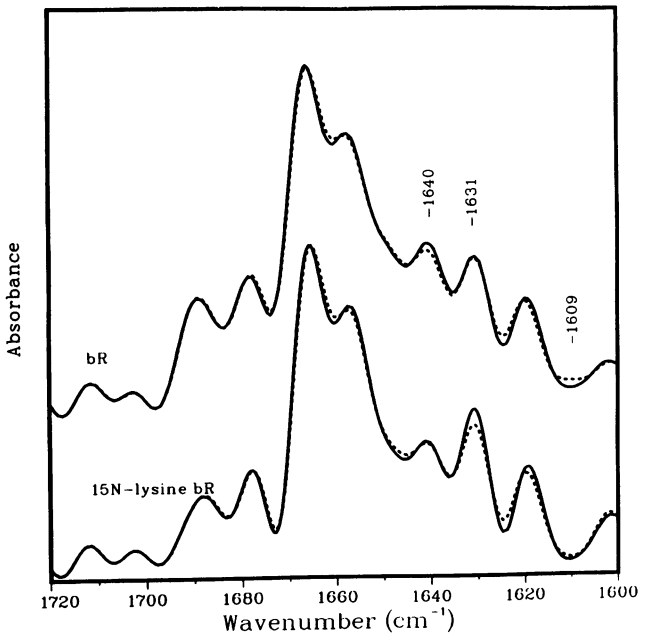


FIGURE 7 PM (*top*) and ϵ-^{15}N-lysine PM (*bottom*) recorded in the bR_{570} state (*solid*) and during illumination with green light which produces the K_{630} state (*dashed*) as described in Earnest et al., 1986. Deconvolution parameters are as in Fig. 5.

three times the photoinduced changes observed in the 1,640 cm^{-1} band (1,630 cm^{-1} in the case of the bR-^{15}N-lysine) can be attributed the $C{=}N$ stretch mode.

DISCUSSION

In this study we investigated the secondary structure of bacteriorhodopsin using a combination of several infrared based techniques. Bacteriorhodopsin offers important advantages as a model system because it can be well oriented (Rothschild and Clark, 1979) and several of the amino acids can be isotopically labeled. In addition, a molecular structure based on electron diffraction and imaging has recently been determined to a resolution of 3.5 Å in the plane of the membrane and ~9–10 Å normal to the membrane (Henderson et al., 1990).

Important features of the FTIR measurements reported here are summarized below:

(*a*) Samples were kept fully hydrated to insure that nonnative structural states induced by drying were not present. While the overall absorbance in the amide I region of dried and humidified films of PM is quite similar, small but significant differences are observed when comparing the deconvolved dry and humidified samples (Earnest, 1987).

(*b*) Samples were cooled from a light-adapted state to low temperature. The primary reason for cooling was to narrow the intrinsic half-width of the individual components of the amide I band, as well as prevent water loss. While cooling could induce a nonnative conformation of bR, it was found, as shown in Fig. 5, that the only observable effect in the amide I region was the anticipated band narrowing. An additional benefit is the ability to trap the light-adapted state of bR (bR_{570}) at low temperature.

(*c*) All measurements were made on oriented samples tilted at 45° relative to the incident beam which was polarized either parallel or perpendicular to the plane of incidence. This allowed the infrared dichroism of the individual resolved components of the amide I and I' (amide I band for the sample in D_2O) to be determined. Previously infrared dichroism has been measured for dried samples of purple membrane (Rothschild and Clark, 1979; Nabredyk et al., 1985) and has been used extensively to study secondary structure orientation in other membrane proteins including rhodopsin (Rothschild et al., 1980), cytochrome-oxidase (Bazzi and Woody, 1985) and proteins from the photosynthetic reaction center complex (Nabedryk et al., 1981; Breton and Nabedryk, 1984). The method has not been previously used, however, in combination with hydrogen/deuterium exchange or in conjunction with resolution enhancement techniques.

(*d*) Photoinduced transitions in the bR photocycle and isotope labeling were used to identify peaks in the resolution enhanced spectrum. This study and an earlier study (Earnest et al., 1988) demonstrate that this method can lead to the identification of infrared bands arising from individual groups in the protein or chromophore. Thus, information in some cases can be obtained about the environment, orientation, and photoinduced alterations of specific groups.

(*e*) Two independent resolution enhancement methods, Fourier self-deconvolution and second-derivative deconvolution were applied to all spectra to verify the existence of deconvolved components of the amide I bands. While only one method is reported here, all results were found to be consistent with second-derivative treatment (Earnest, 1987).

(*f*) Extremely high signal/noise spectra were obtained by collecting several thousand interferograms at a nominal resolution of 2 cm^{-1}. By using a high flow dry-air purge in the sample chamber with a dew-point of −100°C, spectra were obtained free of artifacts due to water vapor. This eliminated many of the spurious narrow band peaks which can lead to erroneous results as previously noted (Lee et al., 1985).

The α-helical structure of bR is resistant to H/D exchange

In agreement with an earlier study on dehydrated purple membrane (Rothschild and Clark, 1979), the present measurements on hydrated purple membrane indicate that the average orientation of α-helices is ~25 degrees from the membrane normal. We are now able to show through our combination of polarized FTIR and H/D exchange measurements that these helices are highly resistant to D/H exchange. This α-helical component of bR most likely corresponds to the ~70–75% slowly exchanging secondary structure previously detected by infrared D/H exchange measurements (Downer et al., 1986) and tritium exchange studies (Englander and Englander, 1977). The inhibition of deuterium exchange on the peptide residues suggests that these residues are buried in the membrane and thus inaccessible to the aqueous exchange media. In addition, α-helices which have strong hydrogen bonds may not be able to undergo rapid H/D exchange.

The relatively high frequency of the amide I band for the α-helices in bR has been previously noted (Rothschild and Clark, 1979, 1980) and has led to the prediction of the unusual α_{II} conformation by Krimm and Dwivedi (1982). One important feature of this model is the predicted splitting of the parallel and perpendicular components of the amide I band which can clearly be seen in the resolution enhanced spectra of Fig. 5, *A* and *B*.

Non-α-helical structure undergoes rapid D/H exchange

In contrast to the bR α-helices, secondary structure associated with the broad band near 1,685 cm^{-1} undergoes rapid D/H exchange in the first 2 h of D_2O exposure (cf. Figs. 2 and 3). The assignment of these rapidly deuterating peptide groups of β-type structure is still tentative, although we would expect beta-turns to be located in the extramembrane surface regions of bR. An amide I frequency higher than 1,670 cm^{-1} is generally interpreted as indicative of either β-turns or β-sheet, the latter also exhibiting an intense mode between 1,620–1,640 cm^{-1}. While we do observe a positive peak at 1,640 cm^{-1} in the D/H difference spectrum, it is smaller than expected and is due in part, as discussed below, to the D/H induced shift of the $C = N$ vibration of the retinal Schiff base.

Evidence against the β-strand model of bR

A key feature of the five α-helix, four β-strand model of bR is the existence of β-sheet type structure with the stands oriented perpendicular to the membrane plane. Such structure would be expected to give rise to linear dichroism in both the 1,680 and 1,640 cm^{-1} regions, with the 1,680 cm^{-1} peak corresponding to the $\nu_{\parallel}(0, \pi)$ mode and the 1,640 cm^{-1} peak corresponding to the $\nu_{\perp}(\pi, 0)$ amide I modes (Fraser and McRae, 1973). For a perpendicular orientation of the strands we would thus expect a positive dichroism at 1,680 cm^{-1} and negative dichroism near 1,640 cm^{-1}.

In fact, a small shoulder at 1,685 cm^{-1} with positive dichroism is found in both the linear dichroism spectrum (Fig. 4) and the resolution enhanced spectra of bR at 250 and 80 K (Fig. 5). However, the expected negative dichroism near 1,640 cm^{-1} is not found. Thus, it is unlikely that a significant fraction of the bR peptides (40 out of 248) could be oriented as predicted by the β-strand model.

It could be argued that the β-strands are oriented such that the transition dipole moment is near the magic angle, however, this would require more peptides in the strand to span the membrane. In fact, much of the relatively small level of absorption near 1,640 cm^{-1} in the resolution enhanced spectra that has previously been attributed to β-strand structure arises from the $C = N$ stretch vibration of the Schiff base, thus limiting the actual amount of β-strand structure which could be present in the protein.

We also note that the 1,685 cm^{-1} band appears to drop in intensity after 2 h of D_2O exposure in both the linear dichroism and resolution enhanced spectra indicating that some of the oriented β-type structure is undergoing D/H exchange. The remainder which represents 50% of the total amount does not change further even after 46 h of additional exposure to D_2O.

In view of the above arguments based on linear dichroism against the existence of a significant fraction of oriented β-strands in bR, it is more likely that the dichroic 1,685 cm^{-1} band arises from peptide groups in a conformation more similar to a β turn. One possible locus for this structure which appears to be both oriented and resistant to D/H exchange is around the three proline groups (Pro-51, Pro-90 and Pro-186) positioned within membrane spanning regions of the bR sequence. Such groups are expected to disrupt the regular hydrogen bonding pattern of the putative α-helices.

Detection of the Schiff base *C=N* stretch mode

In contrast to earlier studies on bR, we assign a significant fraction of the 1,640 cm^{-1} band detected in the resolution enhanced spectrum of bR to the Schiff base ($C = N$) bond linking the retinylidene chromophore to lysine 216. In a related paper (Earnest et al., 1988), a peak at 1,529 cm^{-1}, previously assigned to the amide II band of

β-structure (Lee et al., 1985, 1987), was shown to arise in part from the $C = C$ ethylenic stretching mode of the chromophore. *Thus, evidence based on IR absorption for β-sheet structure in bR which is based on the appearance of peaks in the resolution enhanced amide I and II regions must be treated cautiously because these bands may contain significant contribution from nonpeptide structure.* This work also demonstrates that resolution enhancement of IR spectra, particularly of biomolecules at low temperature, can be used to detect vibrational modes from single groups (see also Rothschild et al., 1989*a*, for additional examples).

CONCLUSIONS

We find evidence for a core of α-helices in bR which are resistant to D/H exchange. In contrast, non-α-helical structure has little net orientation relative to the membrane and undergoes rapid D/H exchange. This data is consistent with a bR structure consisting of surface regions containing β-turns and other weakly hydrogen bonding peptide group conformations while the oriented α-helices are buried in the membrane domain. Preliminary studies using polarized FTIR spectroscopy on the C1 and C2 proteolytic fragments of bR indicates that a majority of the non-α-helical structure is localized on the C1 fragment (residues 72-248) (Earnest, Hunt, Engelman, and Rothschild, unpublished data).

These results are in accord with previous CD measurements which find only limited amounts of β-sheet structure (Nabredyk et al., 1985; Wallace and Teeters, 1987) and as much as 75% α-helices but are not consistent with a model of bR consisting of five α-helices and four β-strands, all oriented perpendicular to the membrane plane. We do, however, detect a small amount of oriented non-α-helical structure which is D/H exchange resistant. It is possible that these regions are localized near proline groups inside membrane spanning regions. One such group, Pro-186, has been found (Ahl et al., 1988, 1989; Mogi et al., 1989) to be essential for maintaining the normal photocycle and proton pumping. In addition, one or more prolines have been recently found to be structurally active in bR (Rothschild et al., 1989*b*). The deviation from α-helical structure inside the membrane spanning regions might be necessary to accommodate specialized protein-chromophore interactions as well as conformational changes involved in proton pumping.

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