RESONANCE RAMAN STUDY OF THE PINK MEMBRANE PHOTOCHEMICALLY PREPARED FROM THE DEIONIZED BLUE MEMBRANE OF *H. HALOBIUM*

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ABSTRACT We report here the Resonance Raman spectrum of a 'pink' membrane (λ_{max} ~495 nm) photochemically generated from the deionized 'blue' membrane (Chang et al., 1985). Comparison of the Raman spectrum of the pink membrane with that of the model compounds, as well as the chromophore extraction data, indicate that the chromophore in the pink membrane is in the 9-*cis* configuration. The Schiff base peak at ~1,652 cm^{-1} shifts to ~1,622 cm^{-1} upon deuteration of the pink membrane, showing that the chromophore is bound to the bacterio-opsin by a protonated Schiff base linkage. The location of the Schiff base peak, as well as the 30 cm^{-1} shift that it undergoes upon deuteration, are quite different from the corresponding values for the native bacteriorhodopsin, suggesting differences in the local environment for the Schiff base in these pigments.

INTRODUCTION

Bacteriorhodopsin, the only protein present in the purple membrane of the extreme halophile *Halobacterium halobium,* is a retinal containing protein. The retinal molecule is bound to the protein, in molar equivalence, by a Schiff base linkage to the ϵ-amino group of Lys_{216}. Light absorbed by this chromoprotein is utilized by the bacterium to create a proton gradient across its cell membrane. This protonmotive force is then utilized to synthesize ATP, which is subsequently used to drive endergonic cellular metabolic processes (see reviews by Stoeckenius and Bogomolni, 1982; Ebrey, 1982).

Light-adapted bacteriorhodopsin, bR_{568}, is a metastable species containing retinal in the all-trans configuration. This species decays thermally to the more stable dark-adapted form, bR_{560}, which contains retinal in both the 13-*cis* and the all-*trans* isomeric forms in roughly equal amounts. It has been found recently that a new species, the blue membrane (λ_{max} ~603 nm), is formed from bR_{560} upon complete removal of all cations by any of a variety of techniques (Kimura et al., 1984; Chen et al., 1984; Chang et al., 1985). The blue membrane, like bR_{560}, also consists of a heterogeneous pigment population, with the chromophore in both the 13-*cis* and the all-*trans* isomeric forms, as revealed by chromophore extraction as well as resonance Raman studies (Smith et al., 1985; Pande et al., 1985). The transition from bR_{560} to the blue membrane is reversible. The reversibility is rather nonspecific for the ion type, but the amount of ions required to cause the change follows the order: $M^+ > M^{2+} > M^{3+}$. The bR_{560}, regenerated from the blue membrane by the addition of ions, is virtually indistinguishable from the native bR_{560}, as judged by the absorption as well as the resonance Raman spectroscopy (Pande et al., 1985).

Recently, while studying the photochemistry of the blue membrane, we found that its absorption maximum shifts from ~603 nm to ~495 nm upon irradiation with light above 640 nm, and the membrane appears pink in color (Chang et al., in preparation). This species is fairly stable in the dark, but converts back to the blue membrane upon illumination with short wavelength light ($\lambda < 500$ nm). In the presence of cations, even though the pink membrane is stable, illumination with short wavelength light results in the formation of the native purple membrane, not the blue membrane.

These results are very interesting, because they show that cations not only regulate the absorption properties of bR, but also determine its photochemical reactions (i.e., the photoproduct of the blue membrane is different from that of the purple membrane). In order to understand the above results and to gain more structural insight, we have used resonance Raman spectroscopy. This technique allows the determination of the isomeric state of the chromophore as well as the nature of the chromophore-protein interactions. Results from our resonance Raman

measurements, in agreement with the chromophore extraction data, show that the chromophore exists predominantly in the 9-*cis* isomeric form in the pink membrane. The chromophore appears to be bound to the protein via a protonated Schiff base linkage. The Schiff base peak position, as well as its shift upon deuteration, are quite different from those observed in bR, suggesting that the Schiff base environment is different in the pink membrane and in its ultimate precursor, the native bacteriorhodopsin.

MATERIALS AND METHODS

The protonated Schiff base of 9-*cis* retinal with N-butylamine was prepared as described previously (Pande et al., 1981). The reaction product was confirmed by its absorption maximum around 440 nm, compared to a considerably blue shifted λ_{max} of the unprotonated Schiff base (~360 nm).

Pink membrane was prepared from the papain-treated blue membrane (Chang et al., 1985) by irradiation with deep red light ($\lambda > 640$ nm) at ~4°C (pH 4.8) using a glass cut-off filter (CS 2-58) in front of a 400 W slide projector. After ~215 min of irradiation, ~80% of the blue membrane was converted to the pink membrane with a λ_{max} around 495 nm.

Deuterated form of the pink membrane was prepared by first concentrating the pink membrane by centrifugation at 4°C and 20,000 *g* for ~4 h, followed by suspension in D_2O. This procedure was repeated two times with the final pellet dissolved in minimum amount of D_2O. Extreme care was taken during this process to avoid contamination from extraneous ions.

The isomeric composition of the chromophores was determined by the retinal oxime extraction method as described earlier (Pande et al., 1985).

Resonance Raman spectra of the pink and the blue membrane were measured at 80 K using apparatus previously described (Pande et al., 1981). At this temperature and the illumination conditions, no spectral changes could be observed even upon prolonged irradiation with the exciting light. This would suggest that the pink membrane does not slowly photoconvert to other species under these conditions. We have confirmed this by low temperature absorption spectroscopy, which shows that 476 nm light does not lead to any photoproducts of the pink membrane at 80 K.

The pink membrane sample was excited with ~5 mW of 476 nm light from an Ar^+ laser (model 165; Spectra-Physics, Inc., Mountain View, CA). Under these conditions the blue membrane contribution to the pink membrane spectrum would be negligible (due to the lower resonance enhancement at this wavelength). The blue membrane spectrum was obtained with ~5 mW of the 488-nm light from the same laser, in order to obtain a somewhat better spectrum, than was possible with the 476-nm excitation. The protonated Schiff base spectrum was measured in a flow system. The temperature of the sample was maintained around 10°C and the sample flow velocity was ~1,500 cm/s. The sample was excited with 476 nm light (~2 mW) as above. Under these conditions, the photoconversion probability in the flow experiment is less than a few percent (Pande et al., 1981) and the Raman spectrum is essentially due to the 9-*cis* protonated Schiff base.

Raman spectra were obtained with a Spex Triplemate spectrometer (model 1877; Spex Industries, Inc., Edison, NJ) with a 1,200 gr/mm grating. The detector system in this set-up consists of a cooled solid state detector (model 1420-2; EG&G Princeton Applied Research Corp., Princeton, NJ) coupled to a controller unit (model 1218; EG&G Princeton Applied Research). This multichannel Raman system, controlled by an LSI-11 computer, can collect data simultaneously in about 700 pixels. This allows a "viewing window" of ~900 cm^{-1}, with a spectral resolution of 8 cm^{-1}, under our experimental conditions. The wavenumber scale of the spectra was calibrated against the Raman lines of toluene.

RESULTS AND DISCUSSION

Upon irradiation with deep red light ($\lambda > 640$ nm), the blue membrane (λ_{max} ~603 nm) partially converts to the pink membrane (λ_{max} ~495 nm). The kinetics of this conversion is very slow as can be seen in Fig. 1, which demonstrates the effect of red light on a sample of the papain-treated blue membrane. The blue membrane does not completely convert to the pink species, even after 215 min of irradiation. Further irradiation did not have any effect on this pigment composition, suggesting that the system had reached a photochemical equilibrium. Since the residual unconverted species has the absorption maximum at ~600 nm and it shows an isosbestic point with the pink membrane spectrum, we believe that it represents the unconverted blue membrane. Our inability to convert this ~600 nm absorbing species completely to the 495 nm absorbing species is probably due to a very much higher quantum efficiency of the back reaction, compared to the forward one. The concentration of this ~600 nm absorbing species in these preparations was estimated to be 20 ±5%. Under identical irradiation conditions, the native (unproteolyzed) deionized blue membrane reaches a photochemical equilibrium which contains greater amounts of the long wavelength absorbing species. Thus, in order to avoid the complications arising in the interpretation of the spectra, we chose to study the pink membrane obtained from the papain-treated blue membrane.

The resonance Raman spectrum of the pink membrane is shown in Fig. 2 *a*. For comparison, we have also included the resonance Raman spectra of the blue membrane that was used to generate the pink membrane (Fig. 2 *b*), and the protonated Schiff base of 9-*cis* retinal (Fig. 2 *c*). One striking feature of these figures is the clear enhancement of

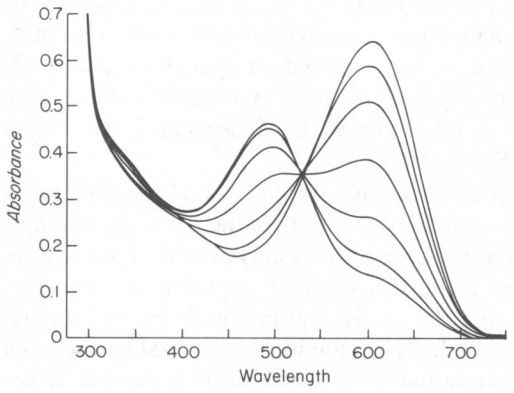

FIGURE 1 Absorption changes in the papain-treated blue membrane (pH 4.8) following irradiation for various times with deep red light ($\lambda >$ 640 nm) at ~4°C, using a glass cut-off filter (CS 2-58) in front of a 400 W slide projector. The sample concentration was ~10 μM. Spectra represent measurements made following irradiation times of 5, 17, 42, 82, 142 and 217 min, respectively. The absorption at ~495 nm increases, at the expense of the ~600-nm peak, with the irradiation time up to almost 217 min. The system appears to reach a photostationary state by this time, since further irradiation did not have any effect on the pigment composition.

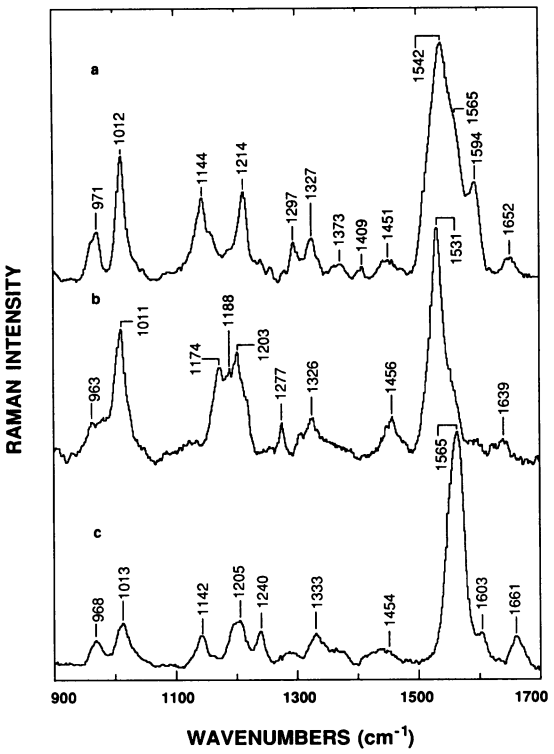

FIGURE 2 Resonance Raman spectra of: (*a*) Pink membrane sample, obtained from papain-treated blue membrane (see text), was excited with ~5 mW of 476-nm line from an Ar^+ laser at 80 K; (*b*) Papain-treated blue membrane was excited with ~5 mW of 488-nm light from an Ar^+ laser, also at 80 K. Slightly higher wavelength was chosen here to improve the spectral quality (see text); (*c*) Protonated Schiff base of 9-*cis* retinal at ~10°C using the flow technique (see text). The sample was excited with ~2 mW of 476-nm light.

the retinal vibrational modes in the 900 to 1,300 cm^{-1} region, relative to the ethylenic mode, when the chromophore is present in the protein matrix, compared to when it is in solution. The salient features of these spectra, as well as their implications, are discussed in detail in the sections that follow.

Ethylenic Region (C=C)

The dominant ethylenic band in the pink membrane spectrum is broad, and is centered around 1,542 cm^{-1}. The absorption maximum of the pink membrane, based on the observed correlation between the absorption maximum and the ethylenic peak position in the retinal based pigments (Aton et al., 1977), is expected to be at ~508 nm, which is in reasonable agreement with the observed value of ~495 nm (Fig. 1). The ~50 cm^{-1} width of the ethylenic peak suggests that the pink membrane may represent a heterogeneous pigment population, rather than a pure species. For example, the width (fwhm) of this band is ~25 cm^{-1} for the dark-adapted bR, which contains an equimolar mixture of the 13-*cis* and the all-*trans* isomeric pigments, compared to the ~14 cm^{-1} band-width of this mode for the light-adapted bR, which contains only the all-*trans* pigment form. The slight hump near ~1,565 cm^{-1} could not be due to the residual blue membrane in the sample, since the dominant ethylenic peak of the blue membrane lies at ~1,529 cm^{-1} (Fig. 2 *b*). Moreover, the contribution of any blue membrane in the sample to the Raman spectrum of the pink membrane, at this excitation wavelength, would be much smaller than its actual concentration, due to the smaller resonance enhancement factors for the blue species (λ ~603 nm). The contribution from the free retinals can also be ruled out because their ethylenic mode is expected to be at 1,580 cm^{-1} or higher (Aton et al., 1977). According to the well known correlation between the λ_{max} and the predominant C=C stretching mode in the retinal systems, noted earlier, the absorption maximum of a species having the main ethylenic band at 1,565 cm^{-1} is expected to be around 435 nm. However, no such species is apparent in the absorption spectrum (Fig. 1), which suggests that if this, indeed, results from the contribution from such a species, its concentration either has to be small, or it is produced in small quantities as a result of a rapid photostationary equilibrium upon irradiation at 80 K. It should be pointed out that a species with a similar absorption maximum (~450 nm) and containing predominantly a 9-*cis* chromophore was observed when the pH of the pink membrane, photochemically generated from the acid-blue membrane, was raised above 6 (Fischer et al., 1981). Since the acid-blue membrane seems operationally indistinguishable from the deionized-blue membrane, the presence of a small amount of this ~450 nm absorbing species in the corresponding deionized pink membrane at pH 4.8 is not inconceivable. Another possibility for the broad ethylenic peak is that two or more C=C modes have unusual Raman intensity. Retinal has five possible C=C vibrational modes, only few of these are intense enough to be normally seen in the resonance Raman spectra of the pigments (see e.g., Callender and Honig, 1977, for a discussion). Thus, the observed shoulder at ~1,565 cm^{-1} could very well result from one of these modes becoming intense upon the formation of the pink membrane. Another, rather intense, band appears at 1,594 cm^{-1}; a similar band at ~1,600 cm^{-1} is also seen in the Raman spectrum of the 9-*cis* visual pigment, isorhodopsin (see e.g., Oseroff and Callender, 1974; Mathies et al., 1977). A relatively strong band at ~1,603 cm^{-1} is also shown by the 9-*cis* protonated Schiff base of retinal (Fig. 2 *c*), while it is absent in the all-*trans* and the 13-*cis* isomeric forms (Pande et al., 1981).

Schiff Base Region (C=N)

The Schiff base peak in the pink membrane spectrum is fairly well defined, and is located at 1,652 cm^{-1} (Fig. 2 *a*). Deuteration of the sample (data not shown) results in a 30 cm^{-1} shift in this peak to 1,622 cm^{-1}. For comparison, the Schiff base peak for the blue membrane and the native bR lie at ~1,639 cm^{-1} and 1,641 cm^{-1}, respectively, (Fig. 2 *b*; Pande et al., 1985), whereas the corresponding values for the protonated Schiff bases of retinals all lie at ~1,661

cm^{-1} (Fig. 2 *c*; Pande et al., 1981). Furthermore, the shifts in the Schiff base mode upon deuteration are ~17 cm^{-1} for bR and ~28 cm^{-1} for the protonated Schiff bases of retinal. The Schiff base peak position, as well as its shift upon deuteration, appear to be very sensitive to the Schiff base environment (Schiffmiller et al., 1985). Although the blue membrane spectrum has not been measured in D_2O due to its extreme lability, comparison of the available data suggests that the Schiff base environment of the native bR changes somewhat upon formation of the blue membrane, but changes even more when the blue membrane converts to the pink species. Besides causing a shift in the Schiff base position, deuteration of the sample also results in some other changes in the Raman spectrum, which we do not understand at the present time.

Finger-Print Region (1,100–1,350 cm^{-1})

The finger-print region of the pink membrane spectrum shows marked differences from that of the blue membrane (compare Figs. 2 *a* and 2 *b*). This region of the spectrum is very sensitive to the chromophore structure, which suggests that the conversion of the blue to the pink membrane is associated with significant changes in the chromophore structure. The peak at 1,144 cm^{-1} in the pink membrane spectrum is absent in the spectra of both the blue and the purple membranes (Pande et al., 1985). Furthermore, the absence of this band in the 13-*cis* and the all-*trans* protonated Schiff bases of retinal (Pande et al., 1981), and its presence at 1,142 cm^{-1} in the 9-*cis* form (Fig. 2 *c*), suggests that this band is characteristic of the 9-*cis* chromophore. These data suggest that the pink membrane contains the chromophore in the 9-*cis* isomeric form. It is interesting to note that a similar band appears at ~1,153 cm^{-1} in bovine isorhodopsin (Oseroff and Callender, 1974; Mathies et al., 1977), which is also a 9-*cis* pigment.

Our chromophore extraction data reveals that the pink membrane contains predominantly (~77%) a 9-*cis* isomeric pigment, in agreement with our Raman data. Two minor components, 15% all-*trans*, and 8% 13-*cis*, were also observed in the extracted mixture. This isomeric composition is similar to that of the pink membrane obtained by irradiation of the acid-blue membrane (Maeda et al., 1980; 1981; Fischer et al., 1981). The all-*trans* and 13-*cis* isomers are in a proportion, 65:35, very close to what we have determined for the blue membrane (Pande et al., 1985) and these isomers represent ~23% of the total retinals present in our pink membrane sample. Since this is close to the amount of the ~600 nm absorbing species we estimate from Fig. 1, we tentatively assume that the *trans* and the 13-*cis* isomers represent unconverted blue membrane. Thus, the 495 nm species probably contains only 9-*cis* retinal.

While bovine isorhodopsin can be regenerated from the 9-*cis* retinal and opsin, no pigment is formed with the 9-*cis* retinal using bacterio-opsin (Schreckenbach et al., 1978). Our ability to photochemically generate a 9-*cis* pigment from the deionized bR shows that ions play a pivotal role in governing the photochemistry of these pigments, presumably exerting their influence on the energetics of the protein structural dynamics.

It is interesting to note that a 9-*cis* pigment with an absorption maximum similar to that of the pink membrane reported here, can also be generated photochemically from the acid-blue membrane (Maeda et al., 1980; Fischer et al., 1981). A similarity in the photochemistry of the deionized- and the acid-blue membranes is not surprising, since they have similar isomeric composition, as well as absorption and Raman spectra (Kimura et al., 1984; Chang et al., 1985; Smith et al., 1985; Pande et al., 1985).

A simplistic picture based on the observed results would suggest that the formation of the blue membrane, either acid or deionized, results in a change in the chromophore binding pocket of the protein, that could now accommodate (or stabilize) a structure such as the 9-*cis* isomer, compared to the more elongated all-*trans* or 13-*cis* form. Thus, the transition state leading to the 9-cis pigment would then be much lower in energy, resulting in a feasible pathway to the 9-*cis* pigment.

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